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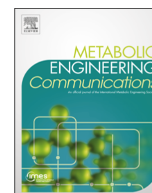

An optimized method for accurate quantification of cell migration using human small intestine cells

Steffen Nyegaard, Brian Christensen, Jan Trige Rasmussen *

Department of Molecular Biology and Genetics – Molecular Nutrition, Aarhus University, Gustav Wieds Vej 10, DK-8000 Aarhus C, Denmark

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ABSTRACT

Quantifying the ability of a compound to modulate cell migration rate is a crucial part of many studies including those on chemotaxis, wound healing and cancer metastasis. Existing migration assays all have their strengths and weaknesses. The "scratch" assay is the most widely used because it seems appealingly simple and inexpensive. However, the scratch assay has some important limitations, as the tool introducing the "wound" might injure/stress the boundary cells and/or harm underlying matrix coatings, which in both cases will affect cell migration. This described method is a Cell Exclusion Zone Assay, in which cell-free areas are created by growing cells around removable silicone stoppers. Upon appropriate staining with fluorescent dyes and microscopically visualizing the monolayers, the migration rate is then quantified by counting the cells (nuclei) intruding the void area left by the silicone insert. In the current study human small intestine epithelial cells were seeded on a physiological substrate matrix to produce collectively migrating monolayers. Different substrates were tested to determine the optimal surface for enterocyte adherence and migration and morphological changes monitored. Recombinant human epidermal growth factor and osteopontin purified from urine were tested to see if the established migration assay produces accurate and reliable migration data with human small intestine cells. The obtained data accurately confirmed that the two bioactive proteins modulate cellular migration in a dose-dependent manner. The presented assay can likely be converted for use with other adherent cell lines or substrate matrices and allows for high throughput, while cost is kept low and versatility high. Co-staining can be applied in order to assay for cell death, different cell types, cell stress and others allowing intricate analysis of migration rate of mixed populations and correction for cell viability.
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1. Introduction

Quick resurfacing is essential for intestinal wound repair in order to regain the ability to function as a protective barrier preventing invasion by pathogenic microorganisms. This is achieved by proliferation and directional migration of enterocytes from the edges of the wound. The quantification of cell migration rate is an important tool to determine the modulatory potential of extrinsic factors on epithelial healing, chronic wounds, villi restitution, cancer cell migration, angiogenesis, embryogenesis and similar types of research (McCormack et al., 1993; Ng et al., 2001; Nobes and Hall, 1999; Stojadinovic et al., 2005; Wang et al., 2008). Different techniques have been employed to quantify cell migration like scratch assays, Boyden chambers and silicone inserts all with separate benefits and drawbacks. The scratch assay is an animal free mimic of wound healing, but consumes many cells and a substantial amount of compound when screening in 6 or 12-well plates. These restrictions make it less ideal for studying primary cultures or patient samples as well as discerning the direct mode of action (molecule-cell, inflammation based migration, paracrine signalling, etc.) (Liang et al., 2007). The Boyden chamber assay (*i.e.* Transwell assay) is a two chamber assay also used for migration studies. It should be noted that the Boyden chamber, especially when coupled with basal membrane extract coating, is technically an invasion assay and commonly used for screening the invasiveness of cancer cells and their expression of metalloproteases necessary to penetrate the extracellular matrix (Marshall, 2011).

The scratch assay is most widely used when quantifying migration rate, as it provides a simple and economical set up in the hands of experienced users. Over time the versatility of the

Abbreviations: (ECM), Extracellular matrix; (Caco-2), human epithelial colorectal adenocarcinoma cells; (FHs-74 int), non-malignant human fetal small intestine cells; (EGF), Recombinant human epidermal growth factor; (ROI), region of interest; (OPN), osteopontin; (BME), Basal membrane extract; (DMEM), Dulbecco's modified Eagle medium; (FBS), fetal bovine serum; (FRET), Förster resonance energy transfer

* Corresponding author.
E-mail addresses: nyegaard@gmail.com (S. Nyegaard), bc@mbg.au.dk (B. Christensen), jatr@mbg.au.dk (J.T. Rasmussen).

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traditional scratch assay has been discussed and several attempts has been made to optimize the assay to accommodate higher throughput, better consistency and enhanced statistics using automation and advanced imaging software (Vogel et al., 2010; Yarrow et al., 2004). Among the disadvantages linked to the scratch assay are that small differences in the protocols for creating scratches convey large inter-lab variance. Scratching can damage the underlying cell-substrate coating and results can be compromised by the release of factors from cells damaged during the formation of the wound by the pin tool (Kam et al., 2008; Staton et al., 2009; Vogt, 2010). Scratching can also be impractical with limited cell or compound resources as previously mentioned.

In order to get precise estimates of cell migration one might wish to reduce adversely contributing factors like cell proliferation, eicosanoid production via cytoplasmic phospholipases, substrate matrix effects, and protein adsorption. Proliferation is commonly negated by starvation in serum free media or by addition of Mitomycin C or similar compounds which arrest cell mitosis irreversibly by DNA crosslinking (Szybalski and Iyer, 1964; Tomasz, 1995). As many proteins adsorb to plastic surfaces to various extents (Andrade and Hlady, 1986), excluding or accounting for the effect of coating the polystyrene with the protein or peptide of interest should be done. Substrate optimization is important for physiological relevance and cell type specificity and should be considered.

It might also be advantageous to minimize phospholipase activation, as this could propagate an inflammatory response that through release of intracellular signal molecules may have impact on cell proliferation and migration (Dennis et al., 1991; Palombella and Vilcek, 1989). Eicosanoid production and release of secretory vesicles is minimized by decreasing cell damage and monolayer disruption which keeps phospholipases deactivated (Arun et al., 2013). Accordingly, as inflammation and wound healing are closely intertwined responses, isolation of the effect of a certain compound on either action is important to disclose the relevant bioactive mechanism in a satisfactory manner (Shaw and Martin, 2009).

The present work was initiated in order to establish a cheap, unbiased and accurate method of quantifying non-malignant human enterocyte migration on biologically relevant extracellular matrix (ECM) as compared to traditional scratch assays. For comparison, scratch assays were performed with human epithelial colorectal adenocarcinoma cells (Caco-2) and non-malignant human fetal small intestine cells (FHs-74 int). The presented assay is based on FHs-74 int and optimized for observing modulatory effects of bioactive proteins on gastrointestinal cell migration rate. We show that treatment with 5 ng/ml epidermal growth factor (EGF) induces migration more than two-fold and show how osteopontin (OPN) induces migration to a similar extent at 5 μg/ml.

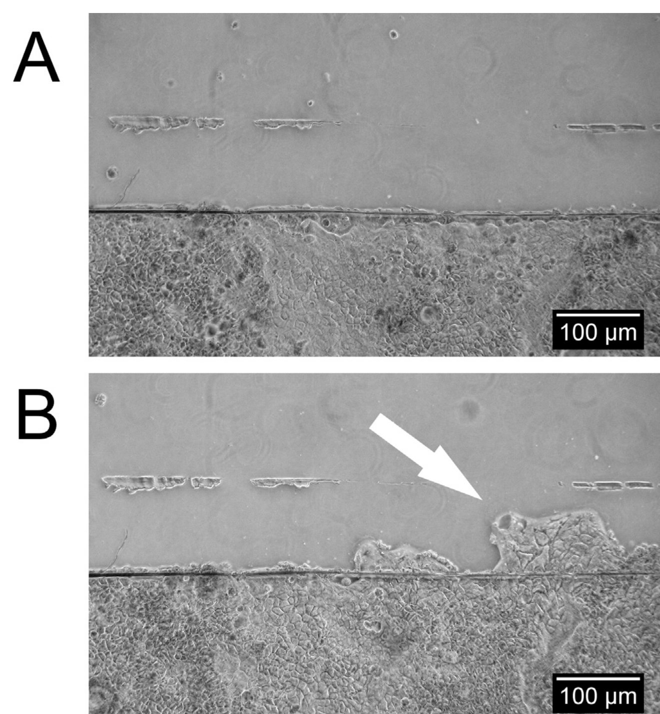

Fig. 1. Scratch wounds and lobed migration. Representative images of Caco-2 cells cut by a razor blade from more than four replicate 6-well plates. Creating wounds using razor blades create high quality wounds as seen in (A). Lobed migration patterns (arrow) were observed after 18 h incubation in starvation media as seen in (B).

2. Materials and methods

2.1. Reagents, cells and software

Fetal small intestine cells (FHs-74 int – ATCC# CCL-241) and tumorigenic colon epithelia cells (Caco-2-DSMZ# ACC 169) were obtained from LGC Standards AB (Boras, Sweden) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and maintained as per manufacturer's instructions. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, recombinant human EGF, SYTO-24® DNA stain (cat# S7559) and CellMask Orange (cat# C10045) were from Life technologies, Denmark. Recombinant human insulin, gelatine from swine skin (cat# G2500), bovine plasma fibronectin (cat# F1141) and propidium iodide (cat# 81845) were from Sigma-Aldrich, Denmark. Basal membrane extract (BME) was obtained from R&D systems (growth factor reduced Cultrex™, R&D systems, Denmark). The Oris migration system was used as donor for our optimized migration assay (Platypus technologies, WI, USA). Clear bottom, 96-well plates were from Nunc, Denmark (cat# 165305). Human OPN was purified from human urine as previously described (Christensen et al., 2008). All chemicals and solutions were analytical grade and endotoxin free when applicable. Images were acquired using Olympus CellB version 3.3 (build 2108) software. Image processing and nuclei counting was done in ImageJ v1.43u using maximum resolution (4140 × 3096 pixel) TIFF files. Microsoft Excel 2010 and Graphpad Prism v5 was used for data handling and statistical analysis.

2.2. Cell culture

FHs-74 int cells were cultured using DMEM + 10% v/v FBS + 1% v/v penicillin/streptomycin + 10 ng/ml recombinant human insulin at 37 °C 5% CO_2 and sub-cultured three times a week at 1:20. The FHs-74 int cells were monitored for morphological changes and experiments carried out between passage three and 15. Caco-2 cells were cultured using DMEM + 10% v/v FBS + 1% v/v penicillin/streptomycin at 37 °C 5% CO_2 and sub-cultured three times a week at 1:10. Experiments were carried out between passage three and 25.

The migration rate was quantified using a modified assay by Platypus technologies. The silicone inserts were rinsed in 70% ethanol, evaporated dry, washed in growth media and mounted in a clear bottom, 96-well plate coated with 50 μg/ml BME protein as per the "thin gel" manufacturer specification. The outermost rows and columns were avoided due to rim effects. 100,000 cells were seeded per well around the inserts to reach rapid confluence. After a 24 h incubation period, the inserts were removed and cell debris

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