In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*

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Published online 1 March 2007; doi:10.1038/nprot.2007.30

The *in vitro* scratch assay is an easy, low-cost and well-developed method to measure cell migration *in vitro*. The basic steps involve creating a "scratch" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the scratch, and comparing the images to quantify the migration rate of the cells. Compared to other methods, the *in vitro* scratch assay is particularly suitable for studies on the effects of cell–matrix and cell–cell interactions on cell migration, mimic cell migration during wound healing *in vivo* and are compatible with imaging of live cells during migration to monitor intracellular events if desired. Besides monitoring migration of homogenous cell populations, this method has also been adopted to measure migration of individual cells in the leading edge of the scratch. Not taking into account the time for transfection of cells, *in vitro* scratch assay *per se* usually takes from several hours to overnight.

INTRODUCTION

The *in vitro* scratch assay is a straightforward and economical method to study cell migration *in vitro*[1]. This method is based on the observation that, upon creation of a new artificial gap, so called "scratch", on a confluent cell monolayer, the cells on the edge of the newly created gap will move toward the opening to close the "scratch" until new cell–cell contacts are established again. The basic steps involve creation of a "scratch" on monolayer cells, capture of images at the beginning and regular intervals during cell migration to close the scratch, and comparison of the images to determine the rate of cell migration.

One of the major advantages of this simple method is that it mimics to some extent migration of cells *in vivo*. For example, removal of part of the endothelium in the blood vessels will induce migration of endothelial cells (ECs) into the denuded area to close the wound[2]. Furthermore, the patterns of migration either as loosely connected population (e.g., fibroblasts) or as sheets of cells (e.g., epithelial and ECs) also mimic the behavior of these cells during migration *in vivo*. Another advantage of the *in vitro* scratch assay is its particular suitability to study the regulation of cell migration by cell interaction with extracellular matrix (ECM) and cell–cell interactions. In other popular methods such as Boyden chamber assays, preparation of cells in suspension before the assays disrupts cell–cell and cell–ECM interactions. In addition, the *in vitro* scratch assay is also compatible with microscopy including live cell imaging, allowing analysis of intracellular signaling events (e.g., by visualization of green fluorescent protein (GFP)-tagged proteins for subcellular localization or fluorescent resonance energy transfer for protein–protein interactions) during cell migration. On the other hand, it is also probably the simplest method to study cell migration *in vitro* and only uses the common and inexpensive supplies found in most laboratories capable of cell culturing.

Although it is developed and more suitable for measuring migration of population of cells, the *in vitro* scratch assay has also been combined with other techniques, such as microinjection or gene transfection, to assess the effects of expression of exogenous genes on migration of individual cells[3–5]. The migration path of individual cells in the leading edge of the scratch is tracked with the aid of time-lapse microscopy and image analysis software. Capturing of an image in the beginning of the experiment with fluorescence microscopy can mark the cells with expression of exogenous gene or downregulation of endogenous genes by RNA interference (e.g., using a GFP marker). By comparing the tracks of these cells with surrounding control cells under the same experimental conditions allows determination of the role of a particular gene in the regulation of directional cell migration using the assay.

There are a number of disadvantages and limitations of the *in vitro* scratch assay compared to other available methods. It does not replace other well-established methods for chemotaxis such as the Boyden chamber assay, as no chemical gradient is established. It takes a relatively longer time to perform than some other methods. One to two days are needed for the formation of cell monolayer and then 8–18 h for cell migration to close the scratch. Last, relatively large amount of cells and chemicals will be required for the assay as it is usually performed in a tissue culture dish. Therefore, it is not a method of choice if the availability of cells (e.g., specialized primary cells that are hard to get in sufficient amount) or chemicals (e.g., expensive reagents) is limiting. The following table summarizes comparisons of the *in vitro* scratch assay with several other methods. Despite these limitations of the method, overall, *in vitro* scratch assay is still often the method of choice to analyze cell migration in a laboratory because it is easy to set up, does not require any specialized equipment and all materials required for the assay are available in any laboratory that performs cell culture.

In this protocol, Steps 1–9 describe the basic method of the *in vitro* scratch assay for measuring migration of cell populations. In Steps 10–13, the method is adopted to track migration of individual cells at the leading edge of the scratch. The latter is suitable to study the effect of particular gene products on cell migration when it is difficult to achieve high efficiency of transfection for exogenous gene expression or siRNA-mediated gene knockdown.

MATERIALS

REAGENTS

- Dulbecco's modified Eagle's medium with supplements (serum, antibiotics)
- Versene (EDTA)with trypsin
- Phosphate-buffered saline (PBS)
- 2 mg ml^{-1} bovine serum albumin (BSA)
- 1 mg ml^{-1} poly-L-lysine stock
- LipofectAmine and PLUS transfection reagents (Life Technologies) (optional)
- Plasmid-encoding GFP or other markers (optional)
- CO_2-independent medium (Optional)

EQUIPMENT

- Tissue culture dishes (60 mm or of other size)
- Razor or extra fine Sharpie marker
- p200 Pipet tips
- Hemocytometer
- Phase-contrast microscope
- Camera
- Fluorescence microscope
- Stage incubator
- CO_2 supply
- Video camera
- Image analysis software

PROCEDURE

Coating of cell culture dishes

1| Coat 60-mm dishes with proper ECM substrates for the cell type to be studied (e.g., for fibroblasts, use 10 μg ml^{-1} fibronectin or 50 μg ml^{-1} poly-L-lysine as a control) by incubating the dishes overnight at 4 °C or for 2 h at 37 °C without rotation or shaking.

2| Remove the unbound ECM substrate and block the coated dishes with 3 ml of 2 mg ml^{-1} bovine serum albumin for 1 h at 37 °C. Then, wash the dishes once with PBS and refill the dishes with 3–5 ml of media before plating the cells. For the particular cell type used, the appropriate amount of serum in the medium during the *in vitro* scratch assay is required to be determined. It is recommended to use a lower percentage of serum than that used in the growth media to minimize cell proliferation, but just sufficient to prevent apoptosis and/or cell detachment.

▲ **CRITICAL STEP** Apart from the serum, if the assay is to study the effects of growth factors or other compounds, these soluble factors should be included in the media before addition of cells.

Passaging the cells in culture

3| Resuspend subconfluent growing cells in a tissue culture dish by washing cells twice with PBS, adding versene containing trypsin, and then mixing cells with medium containing serum. Gently pipette the solution and rock the dish to disperse the cells equally. Take an aliquot from the cell suspension and determine the cell counts using a hemocytometer.

4| Plate cells onto the prepared 60-mm dish to create a confluent monolayer. Incubate the dishes properly for approximately 6 h at 37 °C, allowing cells to adhere and spread on the substrate completely. The required number of cells for a confluent monolayer depends on both the particular cell type and the size of dishes and need to be adjusted appropriately.

Scratch or wound assay

5| The scratch assay can be performed on either native cells (A) or transfected cells (B) to study the effect of specific proteins overexpression (or knockdown) on cell migration.

(A) Scratch assay on non-transfected cells

(i) Scrape the cell monolayer in a straight line to create a "scratch" with a p200 pipet tip. Remove the debris and smooth the edge of the scratch by washing the cells once with 1 ml of the growth medium and then replace with 5 ml of medium specific for the *in vitro* scratch assay.

▲ **CRITICAL STEP** It is important to create scratches of approximately similar size in the assessed cells and control cells to minimize any possible variation caused by the difference in the width of the scratches.

(ii) To obtain the same field during the image acquisition, create markings to be used as reference points close to the scratch. The reference points can be made by etching the dish lightly with a razor blade on the outer bottom of the dish or with an ultrafine tip marker. After the reference points are made, place the dish under a phase-contrast microscope, and leave the reference mark outside the capture image field but within the eye-piece field of view. Acquire the first image of the scratch.

(iii) Place the dish in a tissue culture incubator at 37 °C for 8–18 h. The time frame for incubation should be determined empirically for the particular cell type used. The dishes can be taken out of the incubator to be examined periodically and then returned to resume incubation.

▲ **CRITICAL STEP** Choose a time frame of incubation that allows the cells under the fastest migrating condition to just achieve the complete closure of the scratch.

(iv) After the incubation, place the dish under a phase-contrast microscope, match the reference point, align the photographed region acquired in Step 6 and acquire a second image.

(v) The images acquired for each sample can be further analyzed quantitatively by using computing software of choice. For each image, distances between one side of scratch and the other can be measured at certain intervals (μm) using

Image Pro-Plus software (Media Cybernetics) or a freeware (http://rsb.info.nih.gov/ij/). By comparing the images from time 0 (Step 6) to the last time point (Step 8), (see **Fig. 1**), obtain the distance of each scratch closure on the basis of the distances that are measured by software.

▲ CRITICAL STEP Measure at least 100 readings of distance for each sample and repeat each experiment at least three times. Alternatively, counting of the cells that cross into the scratch area from their reference point at time 0 can determine migration of cells. This method will provide large sample sizes that are easily quantified statistically. Cell counting can be processed by Image Pro-Plus software.

(B) Scratch assay on transfected cells

(i) Plate growing cells at 50–60% confluency for 12–18 h before transfection. Transfect cells with the plasmid encoding the gene of interest (or siRNA) along with a marker plasmid (i.e., GFP) in a 7:1 ratio, or with a vector-encoding GFP fusion protein containing the gene of interest by LipofectAmine and PLUS transfection reagents (Invitrogen). Incubate the dishes at 37 °C until cells reach 100% confluence to form a monolayer.

(ii) Use a p200 pipet tip to create a scratch of the cell monolayer. Wash the plate once and replace with the desired medium. If time-lapse microscopy is used, CO_2-independent media (e.g., HEPES-buffered media) may be required. The time-lapse microscope is used for acquiring the images from the same field automatically. It can be equipped with a stage incubator either with only temperature control or with both temperature and CO_2 control. For the chamber with only temperature control, it is necessary to use CO_2-independent medium during the assay. Nonetheless, it is not very practical to examine cell migration over a period of over 18 h by using the CO_2-independent medium.

(iii) Observe the cells under a fluorescence microscope to ensure that enough cells in the leading edge of the scratch are positively transfected (i.e., as marked by GFP). Create reference markings as described in Step 6 and acquire both phase-contrast and fluorescence images every 2 h for the same scratched region until the scratch completely close or within a desired time frame.

(iv) Determine the rate of cell migration for the transfected cells by the available computing software that measures the distance traveled during the desired time frame (see Step A(v)). It should be noted that the neighboring untransfected cells could be used as controls for the positively transfected cells. It is also useful to draw an imaginary line in the middle of the scratch in the images captured (see **Fig. 2**).

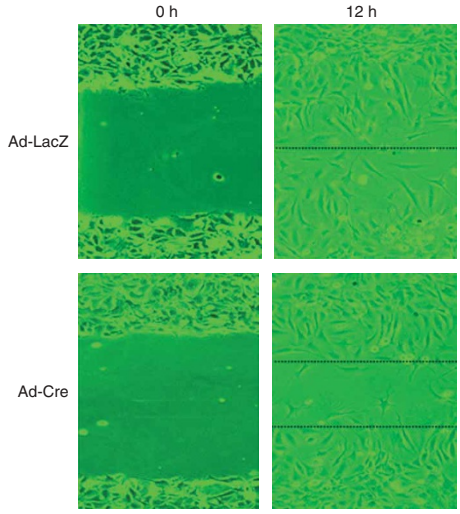

Figure 1 | Analysis of primary EC migration by *in vitro* scratch assay. Primary ECs from floxed FAK mice were infected with Ad-LacZ or Ad-Cre, as indicated. Images were acquired at 0 and 12 h in *in vitro* scratch assay. The dotted lines define the areas lacking cells.

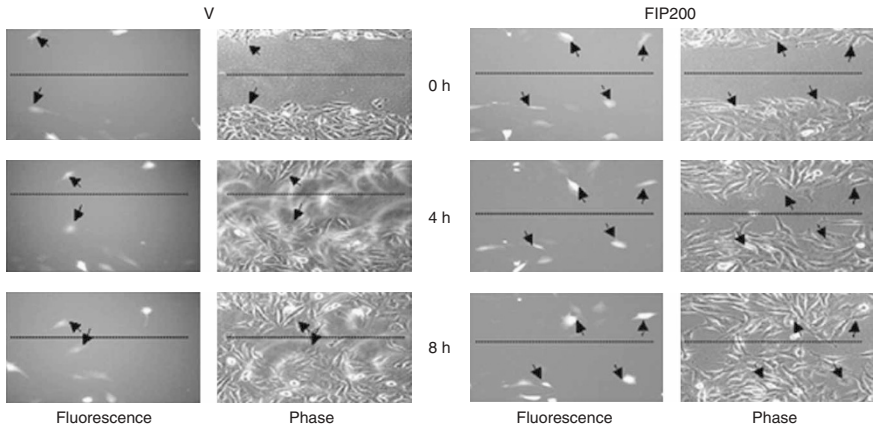

Figure 2 | Measurement of individual cell migration in *in vitro* scratch assay. NIH3T3 cells grown on FN (10 μg ml^{-1}) were co-transfected with the plasmid encoding GFP and expression vector encoding FIP200 or vector alone as control. They were then subjected to *in vitro* scratch assay with images captured at 0, 4 and 8 h after incubation using fluorescence or phase-contrast microscope. The rate of migration was measured by quantifying the total distance that the GFP+ cells (as indicated by arrows) moved from the edge of the scratch toward the center of the scratch (marked by imaginary dotted lines). This figure is reprinted from *Mol. Biol. Cell* **13**, 3178–3191 (2002) with permission from the American Society for Cell Biology.

● TIMING

Step 1: 2 h/overnight (as needed)
Step 2: 1.5 h
Step 3: 30 min
Step 4: 6.5 h
Step 5A(i): 5 min
Step 5A(ii): 5 min
Step 5A(iii): 8–18 h (as needed)
Step 5A(iv): 2–5 min
Step 5A(v): 10–30 min (as needed)
Step 5B(i): 2–3 days (as needed)
Step 5B(ii): 5 min
Step 5B(iii): 12 h or as needed
Step 5B(iv): 10–30 min (as needed)

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1** (**Box 1**).

TABLE 1 | Troubleshooting table.

Steps	Problems	Solution
1	ECM coating is not smooth and shows some strange pattern on the tissue culture dishes	This may happen if the dishes for coating are placed near vibrating sources, such as refrigerators or centrifuges. Remove the vibrating equipment or use another place for ECM coating
3	Cells are clustered in the hemocytometer making it difficult to count the number of cells	Use versene/trypsin mixture instead of trypsin to harvest the cells. Wash cells twice with versene before trypsinizing cells from the dishes. Pipette the solution several times to disrupt the cell–cell adhesion
4	Cells are still round, not attached or spread well after 12 h	The selection of ECM coating may be incorrect for the cells under study. Choose a proper ECM for the cell line and repeat the experiment
5A(i) and 5B(ii)	The edge of the scratch is not smooth because the unscratched part of cell monolayer is detached	Increase the speed of scraping. Wash the cell monolayer with growth medium several times after scraping High amount of DNA or LipofectAmine may be toxic to the cells. Reduce the amount of DNA or LipofectAmine for transfection
5B(i)	Cells are damaged or dead after transfection	Overexpression or knockdown of some genes may reduce cell survival. The researcher should be aware of such potential phenotypes that may influence cell migration
5B(iii)	Cells are damaged or dead	Continuously lighting up the same field for image acquisition may produce heat enough to damage cells. Reduce the intensity of light or use a shuttle that opens only when the image is acquired

ANTICIPATED RESULTS

An example of the *in vitro* scratch assay is shown in **Figure 1,** comparing migration of primary ECs upon deletion of focal adhesion kinase (FAK). Primary ECs from floxed FAK mice were infected with recombinant adenoviruses encoding Cre recombinase (Ad-Cre) to delete endogenous FAK or a control recombinant adenovirus encoding lacZ (Ad-lacZ) for 72 h before the *in vitro* scratch assay. The migration of population of cells is analyzed because of the high efficiency of adenovirus-mediated infection. FBS at 2% (vol/vol) was found to be optimal for the assay, although primary ECs are normally cultured in complete growth medium containing 20% (vol/vol) FBS. VEGF (50 ng ml^{-1}) was included in the assay medium to induce migration of the

BOX 1 | COMPARISON OF DIFFERENT *IN VITRO* MIGRATION ASSAYS

	In vitro scratch assay	ECIS	Boyden chamber	Microfluidics-based system
Cell type suitable for analysis	Adherent cells, monolayer of cells are required	Adherent cells, monolayer of cells are required	Adherent cells	Adherent cells
Sample pool for analysis	Population or individual cells	Population or individual cells	Population of cells	Primarily for individual cells
Ability to track individual cells	Yes	Yes	No	Yes
Gradients	No	Possible for ECM	Yes for both ECM and soluble factors	Yes for both ECM and soluble factors
Sample size in one assay	One condition per tissue culture plate	Up to 96 different conditions for one experiment	Up to 96 different conditions for one experiment	One condition per assay chamber
Complexity of equipment set up	Extremely easy	Commercial plates are available, easy to set up	Commercial chambers are available, easy to set up	Nanofabrication facilities required, difficult to set up
Incubation time before the assay	1–2 days	1–2 days	None	None
Time of the assay	Cell types-dependent (average 14 h)	Cell types-dependent (average 10 h)	Cell types-dependent (average 6 h)	Cell types-dependent (average 4 h)
Data collection and analysis	Cell counting with microscope, and image processing hardware/software	Special-designed devices and software for detection	Cell counting with microscope, and image processing hardware/software	Cell tracking with microscope, and image processing hardware/software
Cost of equipment	No extra cost than routine tissue culture, inexpensive	Commercial instruments and plates, expensive	Boyden chambers and filter membranes, inexpensive	Nanofabrication facilities are required, very expensive

cells. The images at the beginning and the end of a 12 h incubation period were captured, which showed significant migration of the cells toward the scratch. The incubation time was determined at 12 h when the faster moving cells (control, Ad-LacZ-infected cells) were just about to close the scratch.

In **Figure 2**, an example is shown for using the *in vitro* scratch assay to track migration of individual cells in the leading edge of the scratch. This method is used because only a fraction of the NIH3T3 cells were positively transfected as marked by the plasmids encoding GFP. On the left, the cells were transfected with a control vector that did not affect cell migration. As expected, the positively transfected cells (marked by arrows) migrated at the same rate as the surrounding untransfected cells in the leading edge of the scratch. On the right, the cells were transfected with a plasmid encoding FIP200. Analysis of the images showed that the positively transfected cells (marked by arrows) migrated at a slower rate compared with the untransfected control cells in the leading edge of the scratch in the same dish. Furthermore, comparison of the cells transfected with FIP200 (right panels) and those transfected with the vector alone (left panels) also showed inhibition of cell migration by FIP200.

ACKNOWLEDGMENTS This work was supported by NIH grants GM48050 and HL73394 to J.-L. Guan.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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1. Todaro, G.j. *et al.* The initiation of cell division in a contact-inhibited mammalian cell line. *J. Cell Physiol.* **66**, 325–333 (1965).
2. Haudenschild, C.C. *et al.* Endothelial regeneration. II. Restitution of endothelial continuity. *Lab. Invest.* **41**, 407–418 (1979).
3. Etienne-Manneville, S. *et al.* Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* **106**, 489–498 (2001).
4. Fukata, Y. *et al.* Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. *J. Cell Biol.* **145**, 347–361 (1999).
5. Abbi, S. *et al.* Regulation of focal adhesion kinase by a novel protein inhibitor FIP200. *Mol. Biol. Cell.* **13**, 3178–3191 (2002).