

Development of pipette tip gap closure migration assay (s-ARU method) for studying semi-adherent cell lines

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Abstract This work presents a pipette tip gap closure migration assay prototype tool (semi-adherent relative upsurge—s-ARU—method) to study cell migration or wound healing in semi-adherent cell lines, such as lymph node carcinoma of the prostate (LNCaP). Basically, it consists of a 6-well cover plate modification, where pipette tips with the filter are shortened and fixed vertically to the inner surface of the cover plate, with their heights adjusted to touch the bottom of the well center. This provides a barrier for the inoculated cells to grow on, creating a cell-free gap. Such a uniform gap formed can be used to study migration assay for both adherent as well as semi-adherent cells. After performing time studies, effective measurement of gap area can be carried out conveniently through image analysis software. Here, the prototype was tested for LNCaP cells, treated with testosterone and flutamide as well as with bacteriophages T4 and M13. A scratch assay using PC3 adherent cells was also performed for comparison. It was observed that s-ARU method is suitable for studying LNCaP cells migration assay, as observed from our results with testosterone, flutamide, and bacteriophages (T4 and M13). Our method is a low-

cost handmade prototype, which can be an alternative to the other migration assay protocol(s) for both adherent and semi-adherent cell cultures in oncological research along with other biological research applications.

Keywords LNCaP · Migration assay · Semi-adherent cell · Wound healing · Gap closure assay

Abbreviations

s-ARU	Semi-adherent relative upsurge
PC3	Prostate cancer cell line
LNCaP	Lymph node carcinoma of the prostate
ECM	Extracellular matrix
ATCC	American Type Culture Collection

Introduction

Cellular migration is an essential event for normal and abnormal physiological process, such as embryo development, angiogenesis, immune response, wound healing and cancer invasion and metastasis (Albini 1998; Fidler 2002; Wolf et al. 2003; Steeg 2006; Wyckoff et al. 2006; Hulkower and Herber 2011; Justus et al. 2014). Considering the importance of cell migration leading to metastasis in cancer, for example, understanding cancer cell behavior in vitro is an extremely useful tool from clinical research to drug

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discovery (Gupta and Massague 2006; Hulkower and Herber 2011; Kramer et al. 2013). Monolayer wound healing assays have been extensively used for studying changes in cytoskeleton rearrangement, cell-substrate adhesions and degradation, intracellular signaling pathways, nuclear reorganization, and gene expression (Savino et al. 2004; Belo et al. 2016). Also, integrins being the major family of migration promoting receptors (Ridley et al. 2003), their binding to ECM components regulates many signalling proteins involved in cell migration and invasion (Hood and Cheresh 2002). The migration assay is efficient for studying the roles of Ras superfamily of GTPases (Rho protein—Rho GTPases) in cell growth, migration, differentiation and in invasive behavior of carcinoma cells (Keely et al. 1997; Shaw et al. 1997; Allen et al. 1998; Itoh et al. 1999; Nobes and Hall 1999; Roberts et al. 1999; Banyard et al. 2000; DeVries et al. 1999; Burridge and Wennerberg, 2004). It is also reported that migration depends on activation of the ERK pathway and is independent of Ras activation (Anand-Apte et al. 1997; Heldin and Westermark 1999).

Due to its importance, many techniques have been developed for studying cellular migration and various companies have developed commercial kits or inserts for cell migration and invasion assays. The scratch assay or wound healing assay is the most commonly used non-commercial assays for various adherent cell lines, whereas Boyden chamber assayTM or MatrigelTM assay are used for studying the migration and invasion in adherent, semi-adherent and non-adherent cell lines (Kramer et al. 2013). Moreover, many commercially kits or inserts are available to ease such studies, including, Cell CombTM Scratch Assay (Merck Kenilworth, NJ USA.), ORISTM & ORISTM Pro Cell Migration & Invasion Assays (Platypus Technologies, Madison, WI, USA), RadiusTM Cell Migration Assays (Cell Biolabs, Inc., San Diego, CA, USA), CytoSelectTM Wound Healing Assay (Cell Biolabs, Inc., San Diego, CA, USA), Cell migration BiogelTM (Enzo Life Sciences, Inc., NY, USA), Millicell[®] μ -Migration Assay Kit (Merck KGaA, Darmstadt, Germany), QCMTM 24-well colorimetric cell migration assay (Millipore-Merck KGaA, Darmstadt, Germany), and IncuCyte[®] Scratch Wound Assay (Essen BioScience, Inc., Ann Arbor, MI, USA). All these available commercial products are useful and are being used for studying cell migration

and invasion assays. However, there are very few cost-effective options available for studying the migration assays (wound healing assays), especially for semi-adherent (like LNCaP) and non-adherent cell lines.

Considering the need for an economical alternative method for studying semi-adherent cell lines as well as their importance in oncological and other biological research, we report here the developed pipette tip gap closure migration assay (s-ARU method), for studying the migration assays for the semi-adherent as well as adherent cell lines. We applied this method to our studies with LNCaP prostate cancer cell line. The LNCaP cells are androgen-dependent epithelial cells and do not produce a uniform monolayer and are slightly attached to the substrate forming clusters (<http://www.lncap.com/>). They are slow growing and need more incubation time to grow after sub-culturing (<https://www.atcc.org/products/all/CRL-1740.aspx#characteristics>). We hope that our developed low-cost handmade method will prove useful and facilitate the research activities for other adherent and semi-adherent cell lines.

Materials and methods

Cancer cell lines, bacteriophage cultures, and other prerequisites

The prostate cancer-derived LNCaP and PC-3 cell lines (androgen-independent cell) were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). The experiments were performed according to ATCC guidelines. These cells were individually seeded at 1×10^5 cells/well in 6-well culture plates and the experiments were carried out in triplicate. In case of the PC3 cell line, cells were washed with sterile D-PBS (GIBCO/Thermo Fisher). The recommended culture medium used is GibcoTM RPMI 1640 Medium (FBS free) (GIBCO/Thermo Fisher). All procedures were performed inside a 5% CO₂ incubator (Thermo Fisher, Waltham, MA, USA) under strict sterile conditions. The bacteriophage T₄ and M13 were purchased from New England Biolab's Inc (Ipswich, MA, USA). Testosterone and flutamide were purchased from MilliporeSigma (St. Louis, MO, USA).

Preparation of modified 6 well plates

For the preparation of modified well plates, six pipette tips with filter (of 1 ml volume each) were selected and shortened equally to the height of the wells with the help of a sterile cutter (or razor blade, 70% ethanol-treated). The tips were positioned in the center of each well and a drop of silicon glue was added over the filter of the tips. The cover plate was kept over the 6 well-plate with tips positioned at the center with glue at the top of the filter. The cover plate is left to dry. It has to be made sure that all 6 pipette tips have been fixed. For 1 ml pipette tips, the filter position provides a



Fig. 2 Representative image of modified-6-well culture plate prepared for s-ARU method migration assay. Shortened-1 ml pipette tips were fixed in the center of each well-cover using silicone adhesive, for creating a cell-free gap in the plate

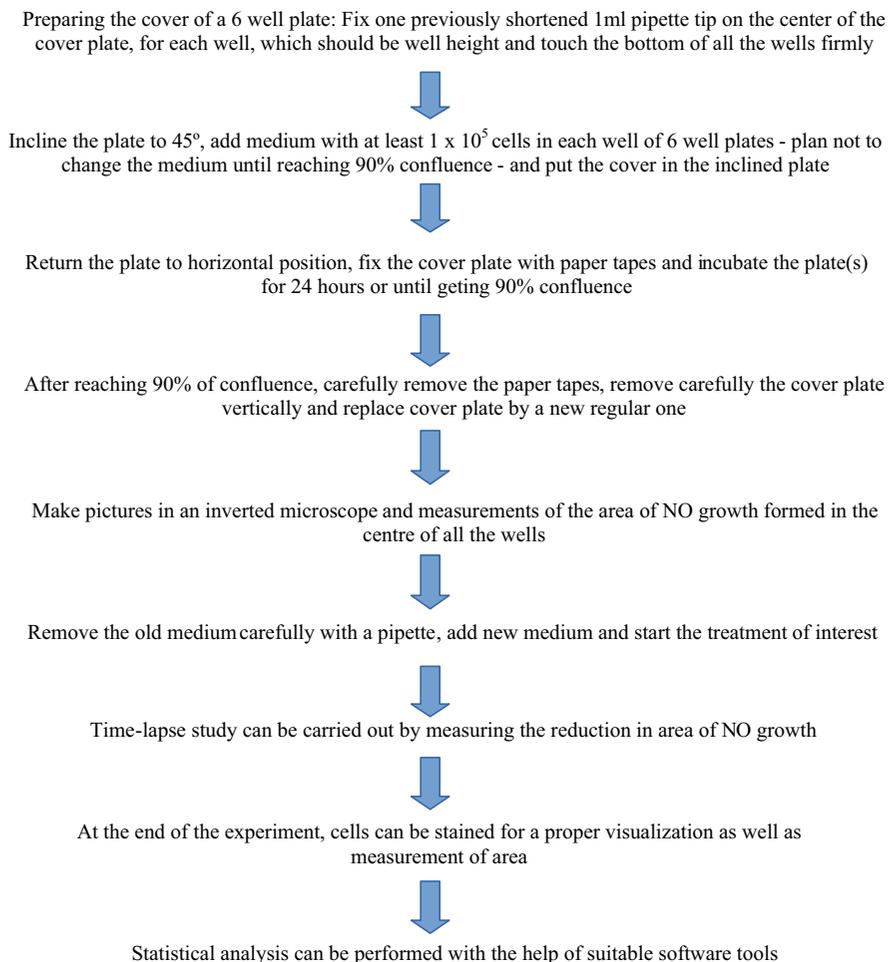


Fig. 1 Flowchart of pipette tip gap closure migration assay (s-ARU method)

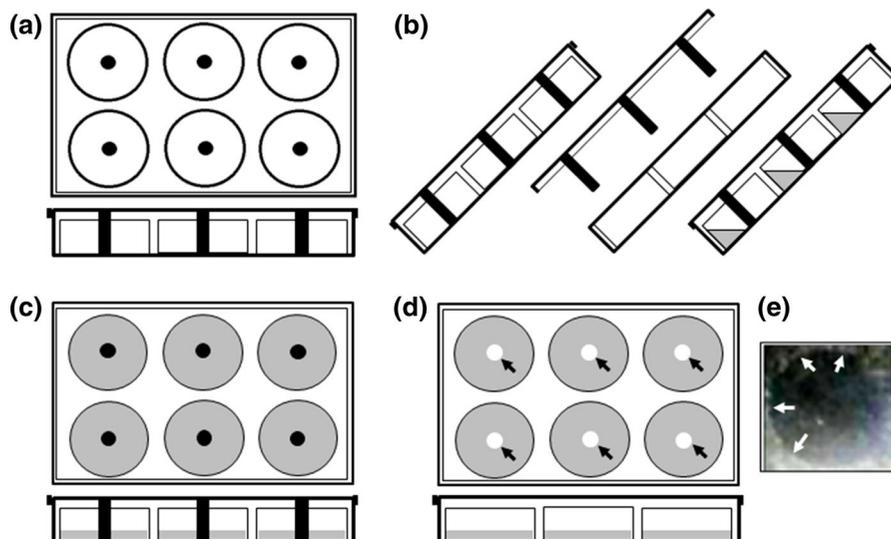


Fig. 3 Schematic representation of modified-6-well culture plate application in the s-ARU method for migration assay using pipette tips as a barrier. **a** Upper and lateral view of the modified-6-well culture plate. **b** Lateral view of the tilted modified-6-well culture plate for application of the medium-containing cells. **c** Upper and lateral view of modified-6-well culture plate in horizontal position, allowing cells to attach and to reach confluence. At this moment, cover plate needs to be fixed with

reference for cutting tips equally and the silicon glue will adjust and correct any difference in height. The directions for plate modifications are given in Figs. 1, 2 and 3. The plates can be UV sterilized to avoid any external contamination.

Inoculation of cells to grow and to reach confluence

With the plates tilted at 45° , RPMI medium mixed with 1×10^5 LNCaP cells were inoculated in each well of the prepared plates. The modified-cover plates with fixed tips were positioned with the plates still tilted at 45° , to avoid entering cells in the cell-free gap. The plates were returned to the horizontal position and the cover plates were carefully fixed with paper tapes. The plates were gently mixed and incubated for 24 h or until getting 90% confluence at 37°C .

Removing the modified-cover plate and measuring the cell-free area

After incubation, the plates were slowly rotated in one direction to detach loosely attached cells and then the

the help of sterile paper tape to restrict the free movement of the cover plate and preventing disturbance of the free-cell borderline. **d** Upper and lateral view of culture plate after cells have reached the confluence. A regular cover plate replaces the modified cover plate. The arrows point to the cell-free gap formed at the center of the wells. **e** Representative view of the cell-free gap formed by LNCaP cells after zone formation under normal condition. Arrows point to the borderline of the cell-free gap

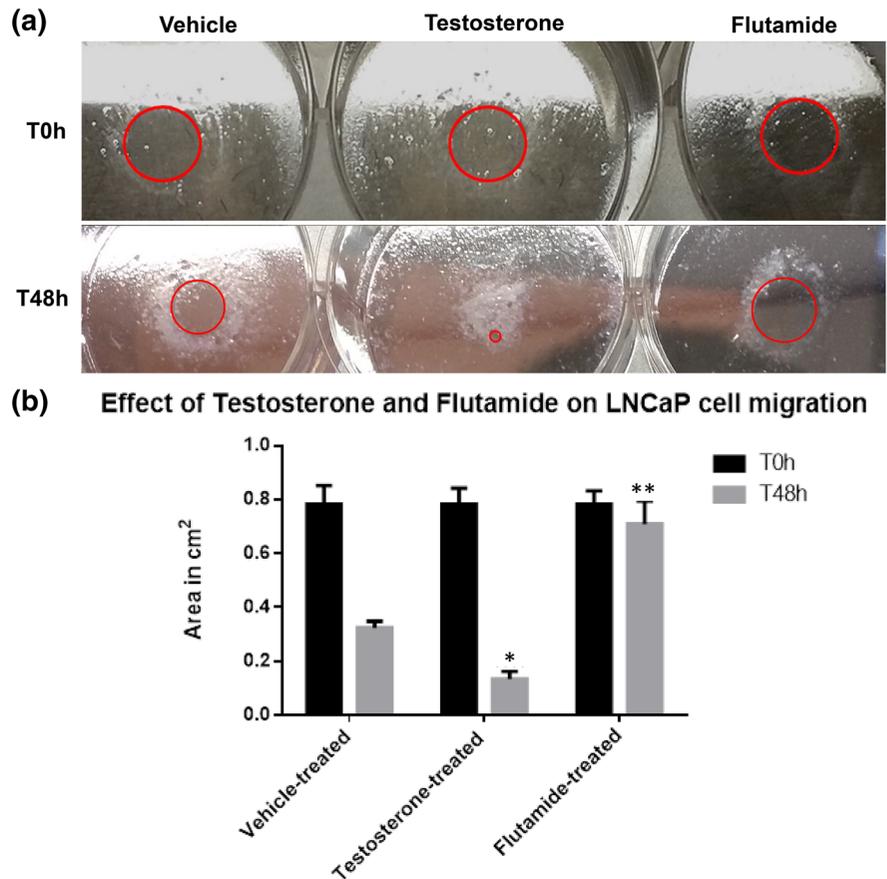
plates were tilted to about 45° to remove the paper tapes and the modified-cover plate. The medium along with suspended detached cells were removed with the help of a pipette (do not use the suction pump as it may disturb the weakly attached monolayer due to suction).

A new medium with the supplements for treatment were added to the wells and regular cover-plates were used for further incubations.

Testing the s-ARU method assay for studying LNCaP cells migration

The s-ARU method was used to study the cell migration assay for LNCaP cells. Confluent LNCaP cells were treated for 48 h with 10 nM of testosterone, 10 μM of flutamide, 1×10^7 pfu/ml of bacteriophage T₄ and M13, separately. For comparison, we also performed the scratch assay for LNCaP and PC3 cell lines. LNCaP and PC3 cells at 1×10^5 concentration were seeded in 6 well-plates. After reaching 90% confluence, scratch assay was performed for the LNCaP and PC3 cells. However, the LNCaP cells exhibited a remarkable irregular borderline after the scratch proceeding, producing significant differences

Fig. 4 a Representative image of LNCaP cells submitted to pipette tip assay (s-ARU method). After 24 h of plating the LNCaP cells and following removal of the pipette tip barrier, a spherical cell-free gap area could be easily identified (red circle), for all treatment, and it represents time zero hour (T0 h) or the beginning of the experiment. **b** Results of the measurements of the cell-free area (red circle) after 48 h (T48 h) of LNCaP cell growth and treatment with vehicle, testosterone and flutamide. The treatment with testosterone highly enhanced the growth and migration of LNCaP cell, while flutamide treatment was highly suppressive as compared with vehicle-treated cells (* means $p > 0.01$; ** means $p > 0.001$). The values are presented as the mean \pm standard deviation. (Color figure online)



in the initial gap area among the experimental groups and replicates. In this sense, it was decided not to proceed with this experiment for LNCaP cells. On the other hands, PC3 cells exhibited a uniform gap area among replicates and experimental groups and were then treated with 1×10^7 pfu/ml of bacteriophage T₄ and M13, separately. After 48 h the image of the wound was digitalized and the measurements of the area were performed.

Statistical analysis

The statistical analysis was performed by using two-way ANOVA with the help of GraphPad Prism 7.04 for Windows (GraphPad Software, San Diego, CA, USA) and ImageJTM software's (Rueden et al. 2017).

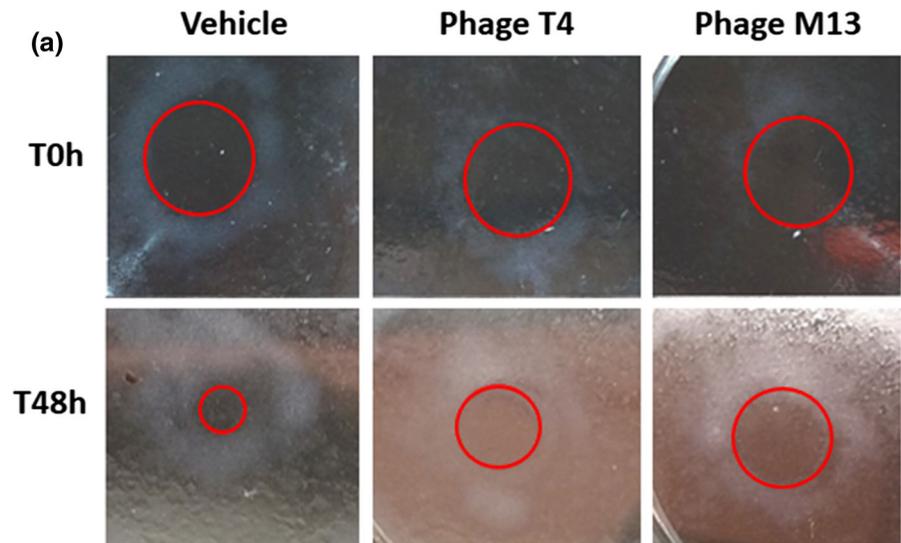
Result

The modified cover-plate with pipette tips attached was effective for creating a cell-free gap area in the center of the 6-well plate for LNCaP cells.

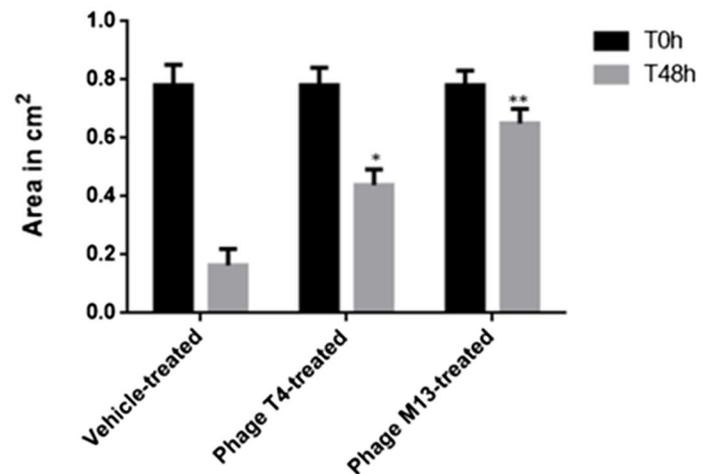
For testing and validating our s-ARU method, we evaluated the effects of testosterone and flutamide, an androgen receptor blocker, on LNCaP cells, which is an androgen-dependent prostate cancer cell line. As expected, testosterone treatment enhanced LNCaP cell growth and migration, whereas, flutamide treatment inhibited LNCaP cell growth and migration (Fig. 4).

We then applied our s-ARU method in another experiment. We were interested in evaluating the effects of bacteriophage T₄ and M13 on prostate cancer cells migration and proliferation, as were previously reported by Dabrowska et al. (2009) for melanoma cells and by Kurzępa-Skaradzińska et al. (2013) for leukemia cells. Here we performed a gap closure assay by exposing LNCaP cell line to two bacteriophages T₄ and M13 using s-ARU method. Our

Fig. 5 Representative image of LNCaP cells cultured using pipette tips as a barrier (s-ARU method). **a** After the incubation of LNCaP cells with pipette tip barrier for 24 h, the cell-free gap could be easily identified (red circle), for all treatment, and it represents time zero hour (T0 h) or the beginning of the experiment. LNCaP cells were exposed for 48 h (T48 h) to bacteriophages T4 and M13 at 1×10^7 pfu/ml concentration. **b** Results of the measurements of the cell-free area (red circle) after 48 h (T48 h) of LNCaP cell growth and treated with bacteriophages T4 and M13. Note that both phages reduced the rate of LNCaP cell migration, in which phage M13 (** means $p < 0.001$) was more effective as compared with the phage T4 (* means $p < 0.01$). The values are presented as the mean \pm standard deviation. (Color figure online)



(b) Effect of Phage T4 and Phage M13 on LNCaP cell migration



method showed that bacteriophage M13 has more anti-migratory effect on LNCaP cells as compared to bacteriophage T4 (Fig. 5).

Since PC3 prostate cancer cell line is an adherent cell, for our study with bacteriophage we performed the scratch/wound healing assay. The wound healing assay performed for the PC3 cells showed that bacteriophage M13 has an anti-migratory effect on PC3 cell lines as compared to bacteriophage T4 (Fig. 6).

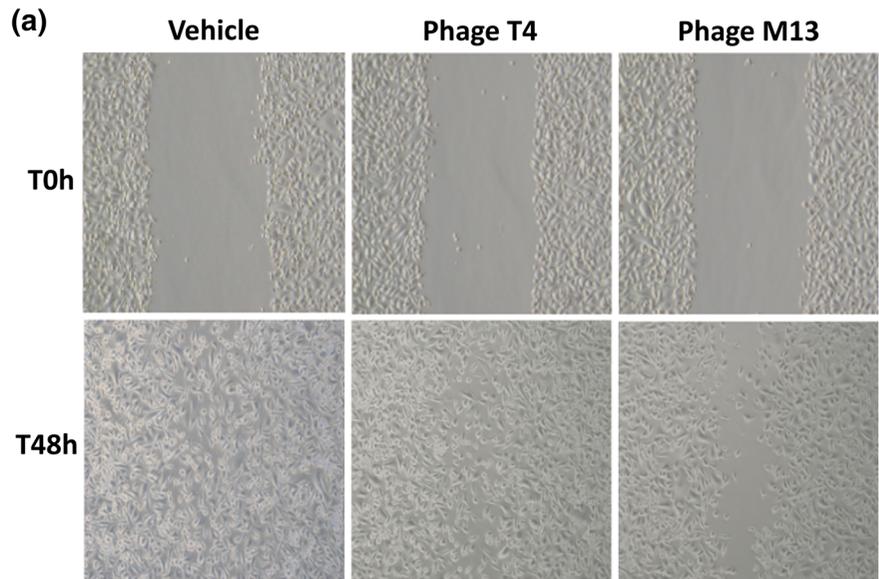
We performed a scratch assay for LNCaP cells also. As expected, LNCaP showed irregular and detached borderline and with remarkable differences among the groups and replicates, due to its semi-adherent nature

(Fig. 7). This aspect did not allow comparison of the initial gap area among the groups and replicates. In this sense, our pipette tip gap closure assay (s-ARU method) seems to be an effective alternative to the scratch assay method for this kind of gap closure assay for LNCaP cell line and others semi-adherent cells.

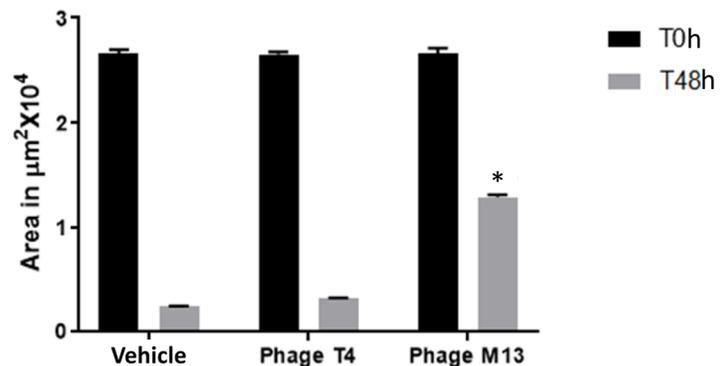
Discussion

Here, we presented a pipette tip assay (s-ARU method) to perform gap closure migration assay for semi-adherent cell lines. Our method can be applied to adherent cells lines as well, alternatively for

Fig. 6 a Representative images of the PC3 cells migration assay by scratch assay method. After reaching confluence, a scratch was performed (T0 h) and cells were exposed to 1×10^7 pfu/ml of bacteriophages T4 and M13 per well for 48 h (T48 h). **b** Results of the measurements of the cell-free area after 48 h. It was observed that phage M13 was effective (* means $p < 0.001$) in slowing down migration rate of PC3 cells and Phage T4 showed no significant effects. The values are presented as the mean \pm standard deviation



(b) Effect of Phage T4 and M13 on PC3 cell migration by Scratch assay



scratching assay. However, further modifications are necessary to minimize the limitations as listed in Tables 1 and 2. Our method seems to be an effective alternative to the available cell migration assays as most of them are ineffective in carrying out such assays for the semi-adherent cell lines and are just limited to adherent cell lines (Table 3) and can be implemented for studying other semi-adherent cell lines (Kramer et al. 2013). Biotech research companies for minimizing plate construction errors and maintaining uniformity in experimental proceedings can also utilize this prototype model for developing

low-cost modified cover-plate for commercial purposes.

Here, using the s-ARU method, we showed that bacteriophage T4 and M13 exhibited the anti-migratory effect on both PC3 and LNCaP cell lines, but bacteriophage M13 appreciably interfered in cell migration rates. The results of this study also corroborate the work of Gorski's group, in which they have studied the effect of bacteriophages on the migration activity of melanom with the help of purified T4 and HAP1 bacteriophages (Dabrowska et al. 2009; Kurzepa-Skaradzińska et al. 2013). They have demon-

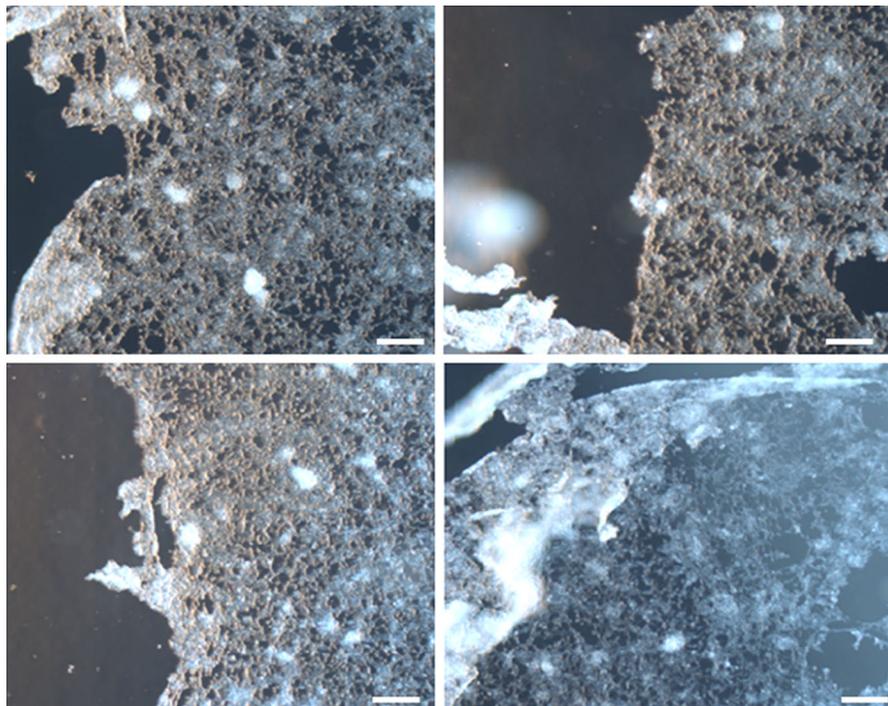


Fig. 7 The representative images of LNCaP cells after scratching for wound healing assay. In the scratch assay the monolayer borderline was disturbed and cells remained freely floating or loosely attached to the base of the plate, creating a

non-uniform and far different gaping area among replicates and experimental groups, and hindering measurements and statistical analysis. Note typical distorted monolayer borderline (scale bar = 100 μm)

Table 1 Advantages and dis-advantages of pipette tip assay (s-ARU) method

Advantages of pipette tip assay (s-ARU method)	Disadvantages of pipette tip assay (s-ARU method)
It can be used for adherent and semi-adherent cell lines	The use of pipette tips for building the modified cover plate unit is prone to human errors
Effective for carrying out toxicity test, drug assay and migration assay	It is time consuming (in the case of semi-adherent cells) when having multiple samples or compounds for analysis
It can be utilized for different microbiological and pathological applications (e.g. testing bioactive compounds against bacterial strains)	In case of semi-adherent cell lines, it is difficult to carry out time-lapse studies with the single plate and needs multiple units to be performed
As compared with the available methods, this method is consistent, reliable and effective	Due to improper operator handling, contamination can be a prime factor affecting final results
It is economical, user friendly and cost effective	Prototype is yet to be established to fulfill all the standards required for global acceptance and commercial production

strated that bacteriophages can interfere in the migration of cancer cells. It would be very interesting to explore further applications of natural and modified bacteriophages in therapeutic approaches for different types of diseases involving cell migration studies.

Our homemade pipette-tip gap closure migration assay (s-ARU method) was developed due to the difficulties associated with studying migration LNCaP cancer cell line because of its semi-adherent nature. Further improvements or progressive development involving this method are most welcome.

Table 2 Troubleshooting for the pipette tip assay (s-ARU method)

Problems	Solutions
Growth in the cell-free gap	- The pipette tip is not properly fixed in the center of the well or - Pipette tip is tilted or - Pipette tip is short
The cell-free area is not spherical/circular	- The cover plate is not fixed properly with paper tape or - The plate was tempered or - The medium and cells have entered under the pipette tip
Contamination	- The pipette tips are not sterile/non-autoclaved or - The pipette tips were not processed within the hood or - The glue/fixative/adhesive used might have contaminated the medium due to over spilling or shaking
The cover plate does not fix the plate properly	- The pipette tips are not homogeneously shortened or - The pipette tip is not properly fixed in the center of the well
Results variation	- The variation in results is mostly due to the density of the cells or - Floating cells occupying the NO growth zone

Table 3 Comparative analysis of different migration assays with respect to pipette tip assay (s-ARU method)

Cell migration methods/ techniques/ assays	Pipette tip assay (s-ARU method)	Trans-well migration assay (Boyden chamber)	Scratch Assay	Electric fence cell migration assay or ECIS	Spheroid migration assay	Capillary tube assays	Time-lapse cell tracking
Cell types supported	Adherent and semi-adherent cell cultures	Adherent, semi-adherent and non-adherent cell cultures	Adherent cell cultures	Adherent cell cultures	Adherent cell cultures	Adherent cell cultures	Adherent cell cultures
Measurement	Area or diameter	Number of cells	Area	Area	Migration area	Migration tube distance	Cell migration path
Complexity	Extremely simple	Extremely simple	Extremely simple	Simple	Simple	Simple	Simple
Cost	Low	High	Low	High	High	High	High
References	–	Kramer et al. (2013) and Poujade et al. (2007)	Kramer et al. (2013), Liang et al. (2007) and Geback et al. (2009)	Kramer et al. (2013) and Cai et al. (2000)	Kramer et al. (2013) and Knupfer et al. (2001)	Kramer et al. (2013) and Young et al. (1999)	Kramer et al. (2013) and Zantl and Horn (2011)

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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