Video Article

A Quantitative Evaluation of Cell Migration by the Phagokinetic Track Motility Assay

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Abstract

Cellular motility is an important biological process for both unicellular and multicellular organisms. It is essential for movement of unicellular organisms towards a source of nutrients or away from unsuitable conditions, as well as in multicellular organisms for tissue development, immune surveillance and wound healing, just to mention a few roles[1,2,3]. Deregulation of this process can lead to serious neurological, cardiovascular and immunological diseases, as well as exacerbated tumor formation and spread[4,5]. Molecularly, actin polymerization and receptor recycling have been shown to play important roles in creating cellular extensions (lamellipodia), that drive the forward movement of the cell[6,7,8]. However, many biological questions about cell migration remain unanswered.

The central role for cellular motility in human health and disease underlines the importance of understanding the specific mechanisms involved in this process and makes accurate methods for evaluating cell motility particularly important. Microscopes are usually used to visualize the movement of cells. However, cells move rather slowly, making the quantitative measurement of cell migration a resource-consuming process requiring expensive cameras and software to create quantitative time-lapsed movies of motile cells. Therefore, the ability to perform a quantitative measurement of cell migration that is cost-effective, non-laborious, and that utilizes common laboratory equipment is a great need for many researchers.

The phagokinetic track motility assay utilizes the ability of a moving cell to clear gold particles from its path to create a measurable track on a colloidal gold-coated glass coverslip[9,10]. With the use of freely available software, multiple tracks can be evaluated for each treatment to accomplish statistical requirements. The assay can be utilized to assess motility of many cell types, such as cancer cells[11,12], fibroblasts[9], neutrophils[13], skeletal muscle cells[14], keratinocytes[15], trophoblasts[16], endothelial cells[17], and monocytes[10,18-22]. The protocol involves the creation of slides coated with gold nanoparticles (Au°) that are generated by a reduction of chloroauric acid (Au^{3+}) by sodium citrate. This method was developed by Turkevich *et al.* in 1951[23] and then improved in the 1970s by Frens *et al.*[24,25]. As a result of this chemical reduction step, gold particles (10-20 nm in diameter) precipitate from the reaction mixture and can be applied to glass coverslips, which are then ready for use in cellular migration analyses[9,26,27].

In general, the phagokinetic track motility assay is a quick, quantitative and easy measure of cellular motility. In addition, it can be utilized as a simple high-throughput assay, for use with cell types that are not amenable to time-lapsed imaging, as well as other uses depending on the needs of the researcher. Together, the ability to quantitatively measure cellular motility of multiple cell types without the need for expensive microscopes and software, along with the use of common laboratory equipment and chemicals, make the phagokinetic track motility assay a solid choice for scientists with an interest in understanding cellular motility.

Video Link

The video component of this article can be found at http://www.jove.com/video/4165/

Protocol

1. Preparation of Gelatin-coated Coverslips

1. Place acid-washed glass coverslips (15 mm in diameter) in a sterile plastic 100 mm dish(es). Place 8-9 coverslips per dish and make sure they are not touching each other or the sides of the dish..

Note: Coverslips, needles and tweezers need to be sterile to eliminate possible contaminating microorganisms, as well as endotoxins that will affect cellular functions, including motility.

2. Weigh the gelatin powder and resuspend the powder in deionized water to make a gelatin solution with a final concentration of 0.5 g/300 ml. Next, the gelatin solution needs to be autoclaved.
3. Pipette 2-3 drops of gelatin (~100-150 ml) onto each coverslip.

Note: Be careful not to allow the gelatin to touch the dish or you will not be able to remove the coverslips from the dish.

4. Bake the gelatin-coated coverslips in the 100 mm dish in an oven at 90 °C for 10 min.
5. Remove excess gelatin by gentle pipetting.
6. Dry the coverslips in the 100 mm dish in the oven at 70 °C for 45 min.
7. Once dry, remove the coverslips from the 100 mm dish using a sterile, endotoxin-free needle and tweezers (the needle is used to gently nudge up the coverslip to make it accessible for the tweezers to gently remove).
8. Place individual gelatin-coated coverslips into separate wells of a 24-well dish.

2. Preparation of Colloidal Gold-coated Coverslips

1. Weigh an appropriate amount of chloroauric acid (tetrachloroauric acid trihydrate; $HAuCl_4 \cdot 3H_2O$) and then resuspend in sterile deionized water to prepare a final 14.5 mM solution (toxic). Prepare 1.5 ml of the solution per 8-9 coverslips.

Warning: Chloroauric acid is harmful if swallowed, causes severe skin burns and eye damage and may cause an allergic skin reaction. Toxic if swallowed.

2. Weigh an appropriate amount of sodium citrate (trisodium dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate; $Na_3C_6H_5O_7$) and then resuspend the powder in deionized water to make a final 0.5% solution. Prepare 1 ml of the solution per 8-9 coverslips.

Warning: Sodium citrate may cause eye and skin irritation. It may also cause respiratory and digestive tract irritation.

3. In a sterile, endotoxin-free beaker combine 1.5 ml of the sterile 14.5 mM $HAuCl_4$ solution and 13.5 ml of sterile deionized water; the result should yield a faint yellow solution. The aforementioned volumes will allow for 8-9 colloidal gold coverslips to be made.
4. While continuously stirring, heat the solution on a hot plate until it begins to boil.

Warning: The heating of the solution should be performed in the fume hood, as vapors can be harmful/toxic. The vapors are destructive to the tissue of the mucous membranes and upper respiratory tract.

5. Remove the beaker from the hot plate.
6. While stirring, add 0.7 ml of the 0.5% sodium citrate solution. The aforementioned volume will allow for 8-9 colloidal gold coverslips to be made.
7. Keep stirring this combined solution for approximately 2 min (Note: The color of the solution will gradually change from faint yellow, to clear, to grey, to purple, to deep purple, before finally reaching a red wine or, preferably, rust color). This product is your colloidal gold solution.
8. Cool the colloidal gold solution in a 10 ml pipette for 1-2 min (in order to keep the gelatin layer on the coverslip from melting when the colloidal gold solution is added) and then add 1.0-2.0 ml of the colloidal gold solution onto each gelatin-coated coverslip previously placed in a 24-well dish(es) (the amount of the colloidal gold solution that you add to the gelatin-coated coverslips depends on how well the gold particles precipitated in the solution - see comments below).

Note: Because there might be variations in the efficiency of gold particle precipitation, it is advised to first add 0.5-1 ml of the colloidal gold solution to the gelatin-coated coverslips and then place the 24-well dish in the incubator for 0.5 hr. After this incubation time, the coverslips should be checked under the light microscope for the appropriate density of the gold particles on the coverslips. See **Figure 1** for 20x microscopy images of examples of too low and too high a concentration of gold particles. See **Figure 2** for 40x microscopy images of an ideal concentration of gold particles (at least for use with monocytes). The optimal density of the gold nanoparticles on the slide should be experimentally determined for each cell type used, as the characteristics of the different cells will dictate their strength of movement on the coverslips. If the concentration of gold particles is insufficient, add an additional 0.5-1 ml of the colloidal gold solution to the gelatin-coated coverslips.

Depending on the cell size, the appropriate concentration of gold particles can vary and, thus will ultimately depend on the size of the cell examined for motility. For example, with human monocytes being small-sized cells (~10-20 μm[28]), a higher concentration of gold particles was required in our analyses. The appropriate distribution of gold nanoparticles provides the researcher with the ability to easily and efficiently distinguish the edges of cellular tracks. A concentration of gold particles that is too high hampers the ability of cells to move and therefore to accurately measure their motility, while a concentration of gold particles that is too low limits ones ability to delineate an accurate track of motility.

Important Note: If the synthesis of gold nanoparticles is problematic, synthesized gold nanoparticles are commercially available in sizes from 5 nm to 400 nm. Additionally, fluorescent microspheres have also been used in studies of cell motility[29]. However, the use of these microspheres requires a fluorescent microscope for the analysis of cell migration.

9. Place the 24-well dish with colloidal gold-covered coverslips in an incubator at 37 °C and incubate from 1 hr to overnight.
10. After incubation, rinse/remove unbound gold by dipping the coverslips (3x) into sterile phosphate buffered saline (PBS; pH 7.4).
11. Place these colloidal gold-coated coverslips in a clean 12-well dish(es) containing PBS.
12. Store these coverslips at 4 °C until ready to use (parafilm the plate before placing in a refrigerator).

Important Note: Always keep the colloidal gold-coated coverslips in some type of liquid/media as the gold particles can flake off from the coverslips if allowed to dry. The colloidal gold coverslips should be used within 2-3 months from the date that they were made.

Quality Control: In a single phagokinetic track motility assay, it is critical to use coverslips that are characterized by a similar concentration of gold particles. This simple point will allow for the quantitative differences in cellular motility between samples to be solely due to the nature of the treatment and not the physical properties of the colloidal gold-coated coverslips. We favor using only coverslips made at the same time in individual experiments, although we have shown that as long as the density of the gold nanoparticles are equal, the use of coverslips from different preparations is appropriate.

3. Analysis of Cellular Motility

1. Place the colloidal gold-coated coverslips in an appropriate cellular media for the cells of interest.
2. Transfer appropriately treated cells onto the colloidal gold-coated coverslips placed in a 24-well dish(es)

Note: The number of cells transferred to the single well needs to be determined for each cell type studied. Ideally, cells need to be equally distributed on the colloidal gold-coated coverslip, which in turn will allow for the statistical analysis of only tracks created by a single cell. If cells are plated at too high a concentration, it becomes highly probable that overlapping tracks of multiple cells will be seen. Overlapping tracks cannot be accurately quantitated and, thus, they cannot be taken into account in the final analyses of the movement of the tested cells. Overlapping tracks as a result of the involvement of multiple cells are usually easily observed; as merged or crossed tracks (in the field of analysis) in which two or more cells can be observed in the same contiguous cleared area.

3. Incubate at 37 °C/5% CO_2 for 24 hr (or for any suitable time; *e.g.* we have analyzed monocyte motility between 6 hr and 24 hr post-treatment and endothelial cells at 12 hr post-treatment). The optimal time frame for determining and measuring cellular motility will vary with the cell type studied and, thus should be experimentally determined for each cell type (a good starting point, however, is 6 - 12 hr post treatment).

Note: If experimental colloidal gold-coated coverslips need to be stored and/or analyzed at a later time, the cells and gold nanoparticles need to be fixed on the coverslips. To accomplish this fixation step; following Step 3.3, first wash coverslips carefully 2 times with 1x PBS (dipping of coverslips is preferable, as a removal of gold nanoparticles from coverslips must be avoided), then use a standard cell fixation method, such as incubation with room temperature 3% paraformaldehyde. After a 15-min incubation, the 3% paraformaldehyde should be removed and the coverslips washed carefully 3 times with 1x PBS. The fixed coverslips can be stored in a refrigerator.

4. Using a light microscope, capture images of the tracks created by a single moving cell (Note: The magnification used to take pictures of cellular tracks will certainly vary depending on the cell type under investigation). Examples of cellular tracks created by non-motile and motile cells on colloidal gold-coated coverslips are shown in **Figure 2**.

Note: The gold nanoparticles of the size used in the phagokinetic track motility assay has been found to be completely nontoxic for cells[30]. If necessary, the viability of the examined cells can be assessed by staining with trypan blue or examined for other markers of cellular viability. One would have to take into account the need for fixation, type of fixation, etc., if this step needs to be undertaken.

5. Using the freely available software, such as ImageJ software (http://rsbweb.nih.gov/ij/) or NIH Image (http://rsb.info.nih.gov/nih-image/), both developed at the National Institutes of Health, or ImageTool (http://ddsdx.uthscsa.edu/dig/itdesc.html) developed at the University of Texas Health Science Center at San Antonio, the average area (in arbitrary units) of colloidal gold cleared by 10-20 or more cells (per sample) is determined for each experimental arm from the captured images. Statistics can then be performed on the collected results. For example, results can be plotted as means ± the standard errors of the means (SEM) with Student's *t* tests performed, and a *P* value of <0.05 used as the measure of statistical significance between samples. **Figures 3 and 4** show steps in the analysis of the area of colloidal gold cleared by the cell.

Representative Results

Shown is an example of pictures taken under a light microscope showing a track area cleared by a single cell (a monocyte from our experiments is shown in **Figure 2)**. Non-motile cells create characteristic small, oval or circle-shaped tracts around themselves indicating a low basal level of movement for these unstimulated cells (**Figures 2A and 2B**). In contrast, highly motile cells [in our system, human cytomegalovirus (HCMV)-infected cells] are characterized by a directional movement shown in **Figures 2C and 2D** as elongated track areas. After obtaining multiple pictures of track areas using an inverted microscope with a 40x objective, the total track areas were marked using the ImageJ software **(Figure 3)**; similar results can be obtained with other software applications. We favor the ImageJ software because it is free and easy to use. Motile cells do not create standard, geometrical shaped tracks during their movement; thus, a convenient way to mark the area to be analyzed is to select the freehand tool in the ImageJ software in order to mark the shape of track created by the moving cell (**Figure 3**). Having delineated the track area to be analyzed, the quantitative measurement of the data is performed by clicking on "Analysis" in the ImageJ menu bar and choosing "Measure" from the pull down menu. A sample of our results in arbitrary units obtained by analyzing the cellular track areas collected from the data presented in **Figure 3** is shown in **Figure 4A**. The results collected from the ImageJ software can then be analyzed in a spreadsheet of choice to allow for the calculation of the average track area cleared per cell as shown in **Figure 4B**.

In our experience, the most problematic step of the protocol is the production of the colloidal gold-coated coverslips; the issue of concern is the generation of coverslips with uniform coverage of gold nanoparticles (issues related to this concern are discussed in **2.8** above). The appropriate, uniform coverage of gold nanoparticles on a coveslip is presented in **Figure 2**. Coverage that is too dense (**Figures 1A and 1B**) or too sparse (**Figures 1C and 1D**) will prevent cell migration or alter the reproducible analysis of the size and the shape of the track area. The second key point as discussed in **3.2** above, is the density of the plated cells and the need to prevent overlapping or merged tracks from multiple cells.

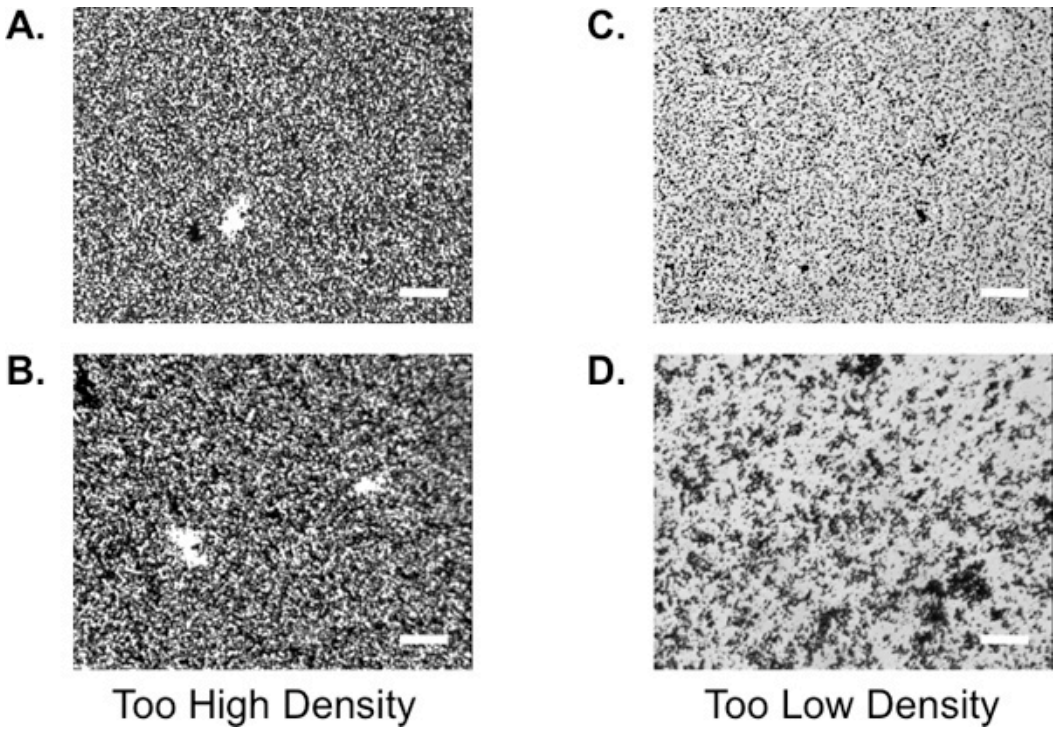

Figure 1. Potential Problems with the Density of Colloidal Gold on the Coverslips. Shown in this figure are examples of colloidal gold-coated coverslips with either a too high (Panels **A** and **B**) or a too low (Panels **C** and **D**) density of gold nanoparticles. Both of these examples will lead to poor data collection and statistical analyses. Note: Inappropriate concentrations of chloroauric acid or sodium citrate, as well as prolonged boiling of the reaction solution, may cause these undesirable results. Pictures were taken by using a 20x objective. Note: Panel **D** also depicts undesirable aggregates of gold nanoparticles on the glass coverslips. Size bar corresponds to 50 µm in length.

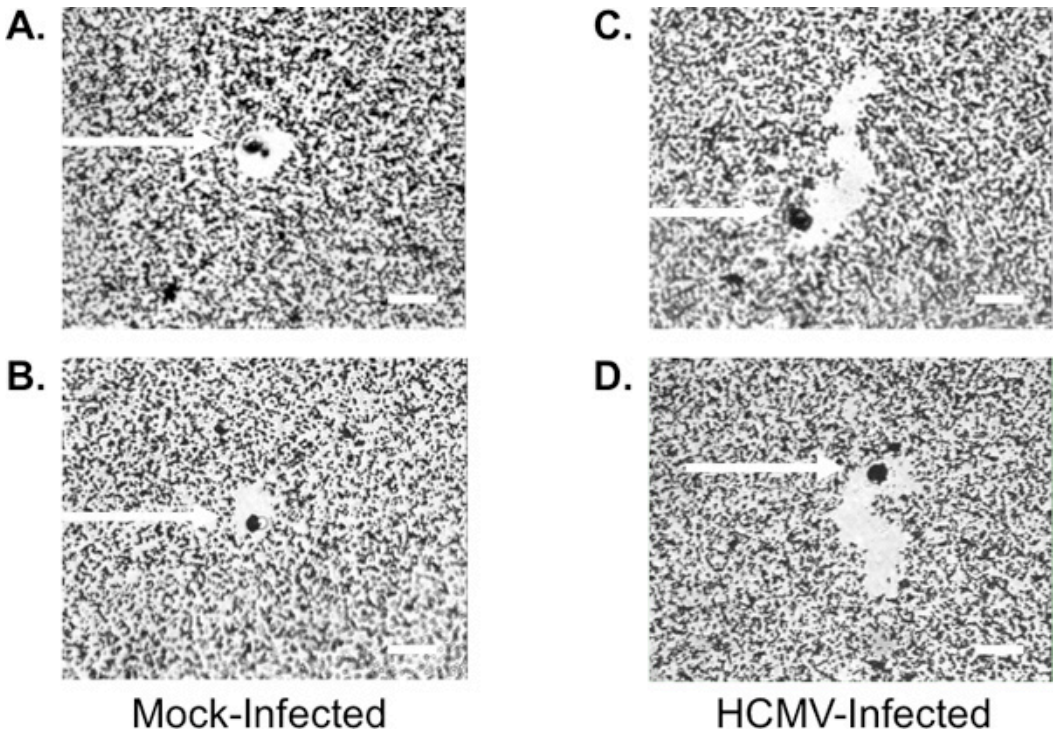

Figure 2. Examples of tracks created by non-motile (Panels A and B) and motile (Panels C and D) cells on colloidal gold-coated coverslips. Virus-free media (mock-infected) or media containing viral particles (HCMV-infected) were added to isolated peripheral blood monocytes[10,18-22]. Monocytes were incubated at 37 °C/5%CO_2 for 1 hr and then plated onto colloidal gold-coated coverslips for 24 hr at 37 °C/5%CO_2. Pictures were taken by using a 40x objective. White arrows mark monocytes in their final location. As we have demonstrated previously[10,18-22], uninfected monocytes are characterized by a low basal level of motility, while HCMV-infected cells show characteristics of high motility. Size bar corresponds to 25 μm in length.

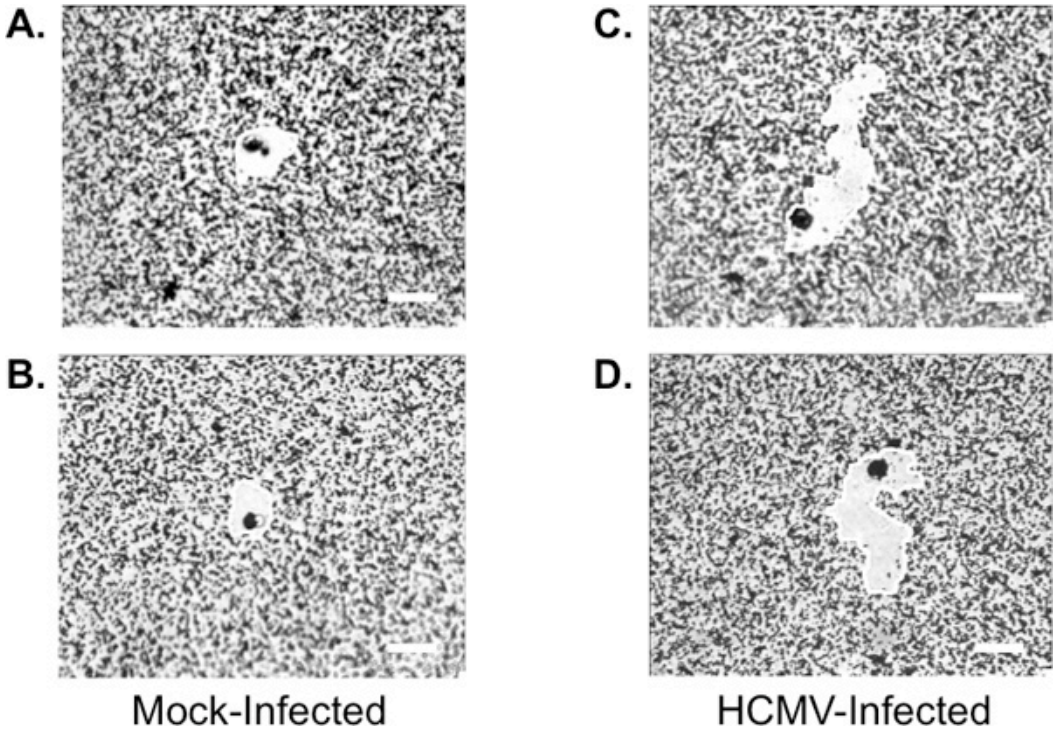

Figure 3. Marking the Area of the Cellular Tracks Using ImageJ software. Examples shown are snapshots of tracks, in which the Image J software was used during the analysis of the tracks created by non-motile (Panels **A** and **B**) and motile (Panels **C** and **D**) cells on colloidal gold-coated coverslips (Note: Tracks are circled by a white line using the ImageJ freehand tool). The sample pictures, the same as those shown in **Figure 2**, were used in this figure to measure track areas cleared by a single cell. These are representative images of mock- and HCMV-infected monocytes, however, usually 10-20 images are analyzed per sample. Size bar corresponds to 25 μm in length.

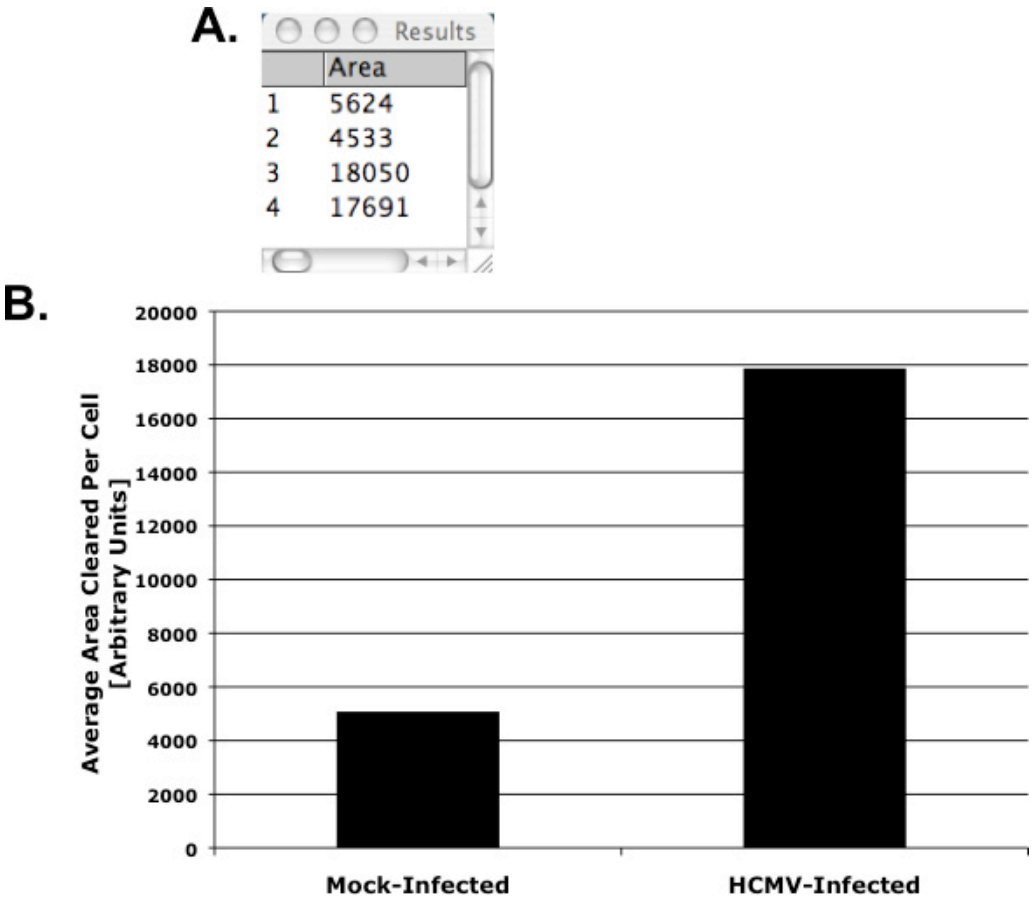

Figure 4. The Quantitative Evaluation of Cell Motility Using ImageJ Software and Spreadsheet. A) Measured track areas cleared by cells, as depicted in the sample pictures (**Figures 2 and 3**), using the ImageJ software. The calculated results **#1**, **#2**, **#3** and **#4** correspond to Panels **A**, **B**, **C** and **D** in **Figures 2 and 3**, respectively. **B)** A graphical representation of average areas (means; in arbitrary units) cleared by cells and analyzed by the ImageJ software. In this example, the standard error of the mean is not shown, because in the example an insufficient number of replicates is analyzed. Note: The ImageJ software provides the measured area of tracks in arbitrary units; therefore, it is critical to compare pictures of tracks taken under the same magnification. The simplest method to compare results from separate experiments is to compare fold changes between examined samples.

Discussion

The phagokinetic track motility assay presented in this article is a simple and highly effective method for quantitative analysis of cell migration. Because multiple cell types can be analyzed[9-17], this method has the potential broad usage across multiple disciplines. The use of colloidal gold-coated glass coverslips allows for the measurement of a track area cleared by a moving cell. The assay can measure the effect of different stimuli (*i.e.* growth factors, purified ECM ligands, viruses, bacteria) on cell motility. Additionally, a lack of the requirement for cell fixation allows for monitoring of cell migration at different experimental time points. Moreover, the assay requires only a standard light microscope, a standard camera using a CCD (charge coupled device) image sensor and freely available software. Therefore, the sophisticated microscopes and cameras are not needed, nor are special software suites. Even though the assay is simple in design, it is a powerful tool to statistically study the motility of multiple cell types under a multitude of experimental conditions. For example, in this regards, the assay can be used to effectively study a small number of experimental samples, as well as to effectively serve as a high throughput screening assay. Because the coverslips can be examined unfixed, the assay can also allow for the analysis of moving cells over time without the need for a microscope with an enclosed humidified, heated CO_2 incubator. Additionally, the phagokinetic track motility assay is helpful in studying a motility of cells not amenable to a time-lapse imaging, as a photobleaching of fluorophore-stained cells is less of a concern in the colloidal gold-based motility assay. The quantitative evaluation of track areas cleared by a single cell is achieved by using the freely available ImageJ software, although other software can be used. Moreover, the overall graphical and statistical analyses obtained only require a basic knowledge of calculations, spreadsheets, and statistics.

It should be noted that the original method described the use of bovine serum albumin (BSA) to cover the glass coverslips[9]. However, the use of BSA was found to be unreliable as an agent to allow for the uniform gold colloid monolayer[27]. The substitution of gelatin for BSA, as the coverslip's coating, increased the adherence of gold nanoparticles to the coverslip. Additional modifications introduced by Scott *et al.*[27] to the protocol greatly improved the quality of the gold nanoparticle coating. Nevertheless, we found, based on Turkevich *et al.*[23], that the use of sodium citrate as a reducing agent created the most uniform monolayer of gold nanoparticles. Therefore, we favor this method and as such, it is described in our protocol.

To allow for a high degree of reproducibility of the assay, it is important to strictly follow the volumes and temperatures indicated in the protocol, as deviations can strongly influence the final results. As discussed above, one of the technical hurdles in the use of the phagokinetic track motility assay involves the production of the gold nanoparticles in the solution and then in obtaining an appropriate uniform concentration of the gold nanoparticles on the coverslip. Thus, even though the gold nanoparticle precipitation step is reproducible, we place an emphasis on assessing the color of the final solution of precipitated gold nanoparticles. Additionally, this is our rationale for only adding half of the normal volume of the final solution onto each coverslips and, then after 0.5 hr, evaluating the density and uniformity of the gold particles on the coverslips (with the ability to add additional solution, if needed later). These precautions are taken to avoid creating a state where the coverslips show either too high (**Figures 1A and 1B**) or too low (**Figures 1C and 1D**) a concentration of gold nanoparticles on glass coverslips.

Overall, the phagokinetic track motility assay is a straightforward method to analyze cell movement. It provides a snapshot of the distance (via the final track area) that a cell moved over a defined time frame. Furthermore, the assay is very amenable to multiple stimuli; that is, examined cells can be tested under a variety of treatment options. For example, in our monocyte experiments, we have tested the motility of virally-infected, cytokine-treated, growth-factor-treated, mitogen-treated, and LPS-treated monocytes. Furthermore, we have tested these activated monocytes under a variety of inhibitors/inhibitory conditions to functionally examine the role various biochemical/molecular pathways play in monocyte motility. Unlike a wound-healing assay[31], measuring a cumulative effect of cell proliferation and migration, or a Boyden chamber (trans-well) migration assay[32], allowing a bulk analysis of cell chemotactic motility, the phagokinetic track motility assay evaluates the migration of single cell. However, if there is a need for a bulk chemotactic effect to be measured, a three-dimensional migration/invasion to be assessed, a more detailed analysis of a cellular movement, or the analysis of cell movement under *in vivo* conditions, this protocol would not suffice and the use of additional methodology, such as a trans-well migration assay, a matrigel invasion assay[33], live time-lapsed microscopy/photography[34,35], etc. would need to be considered.

The phagokinetic track motility assay is a simple, quantitative evaluation/comparison of an ability of cells to migrate without a need for using expensive microscopes and software in the analyses. It is addressed to researchers in need of a quick and easy, but reliable, method to quantify cell motility, offering a possibility of a simple high-throughput assay.

Disclosures

No conflicts of interest declared.

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