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Statistical analysis of cell migration in 3D using the anisotropic persistent random walk model

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Abstract

Cell migration through three-dimensional (3D) extracellular matrices is critical to the normal development of tissues and organs and in disease processes, yet adequate analytical tools to characterize 3D migration are lacking. The motility of eukaryotic cells on 2D substrates in the absence of gradients has long been described using persistent random walks (PRW). Recent work shows that 3D migration is anisotropic and features an exponential mean cell velocity distribution, rendering the PRW model invalid. Here we present a protocol for the analysis of 3D cell motility using the anisotropic persistent random walk model. The software, implemented in MATLAB, enables statistical profiling of experimentally observed 2D and 3D cell trajectories and extracts the persistence and speed of cells along primary and non-primary directions and an anisotropic index of migration. Basic computer skills and experience with MATLAB software are recommended for successful use of the protocol. This protocol is highly automated and fast, taking less than 30 minutes to analyze trajectory data per biological condition.

Summary

This protocol enables the statistical analysis of cell motility in 2D and 3D microenvironments using the anisotropic persistent random walk model, providing a new tool to characterize 3D cell migration.

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AUTHOR CONTRIBUTIONS. A.G. conducted the experiments. P.W. developed, tested, applied, and validated the APRW model. A.G., P.W., and D.W. designed the experiments, analyzed the results, and wrote the paper.

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Supplementary Information

Combo PDF - Supplementary Methods - Cell culture and motility assays Supplementary Software - contains MATLABscripts

Supplementary Data - contains example excel sheets

Keywords

3D motility; cancer; anisotropic persistent random walk model; APRW; PRW; persistent random walk model; trajectory; mean squared displacement; autocorrelation function; angular displacement distribution; cell migration; cell motility; cell trajectory

INTRODUCTION

Cell migration is one of the basic cell functions that underlie life. Eukaryotic cell migration is a complex process that is tightly regulated through hierarchical molecular pathways and is critical to the normal development of organs and tissues^{1–3}. Onset of migration in an adult organism or ill-regulated cell motility are often associated with human diseases, including cancer metastasis^{4, 5}, immunological responses⁶, and wound healing⁷. An increasing amount of basic and translational research relies on cell motility assays to obtain information about changes in cell migration in response to chemical, mechanical, and genetic perturbations. Hence a rigorous protocol to analyze experimentally measured cell migratory patterns is critical.

Cell motility is a stochastic process and extraction of relevant parameters fully describing this motility relies on understanding random patterns of cell movements, typically measured under time-resolved light microscopy. In the absence of symmetry-breaking gradients (such as directed cell migration towards chemoattractant sources), the motility of bacteria and eukaryotic cells has long been described in terms of random-walk statistics. The persistent random walk (PRW) model has been widely used to characterize eukaryotic cell motility on flat 2D dishes. This model describes the trajectory of cells as a succession of uncorrelated movements of duration equal to the persistence time. From fits of the mean squared displacements (MSDs) of the cells with the PRW model^{8–12}, 2D motility would be fully characterized with just two parameters: cell speed and persistence time.

However, fits of MSDs do not rigorously test several underlying assumptions of the PRW model. These assumptions include: a Gaussian distribution of velocities, an exponential decay of the velocity correlation function, an isotropic velocity field, and a flat distribution of angles between cell movements at long time scales¹³. There is a practical challenge to testing these assumptions directly at the single-cell level: the inherently limited timespan of a cell, which is equal to its cell cycle. An alternative test of the assumptions underlying the PRW model has been to obtain statistical characteristics of cell motility from population-averaged MSD profiles to obtain the required resolution. However, recent motility studies have shown that, at even at the ensemble-averaged level, a Gaussian distribution of velocity does not occur for a wide range of cell types crawling on 2D substrates^{14–16}, questioning the validity of the PRW model to analyze cell migration, even on 2D substrates.

Aside from this dilemma, accumulating evidence suggests that cell migration in 3D extracellular matrices (ECMs), a more physiologically relevant condition, requires mechanisms of force generation, cell-matrix adhesion, and signaling pathways that are distinct from those required for 2D migration^{17–23}. Mesenchymal cell migration *in vivo* often forces cells to remodel, exert pulling forces on, and move through a 3D collagen I-rich

matrix, the main structural protein of connective tissues²⁴. Migration on 2D collagen-coated dishes is driven by actomyosin contractility of stress fibers between large focal adhesions and the formation of a wide lamellipodium terminated by thin filopodial protrusions at the leading cellular edge^{3, 25}. The same cells in a collagen-rich 3D matrix display highly dendritic pseudopodial protrusions that rely both on actomyosin contractility and microtubule dynamics^{19, 26}. Further, 3D cell migration is tightly associated with the expression of metalloproteinases (MMPs)²⁶ and physical properties of the 3D matrix^{5, 18, 19} which are dispensable in 2D migration.

Despite the fact that cells adopt fundamentally different strategies to migrate on 2D substrates and in 3D matrices, the PRW model remains frequently used to analyze patterns of migration in 3D matrix because a suitable model for 3D cell migration has been lacking. This paper provides a detailed protocol to analyze cell migration in 2D and 3D microenvironments.

Development of the protocol

In recent work²⁷, we rigorously examined the stochastic motility of HT-1080 human fibrosarcoma cells embedded in 3D collagen matrices using a set of statistical functions, including the MSD, the velocity autocorrelation function (ACF), the probability density function of cell displacements (PDF-dR), the probably density function of angular displacements (PDF-d θ), and the velocity profiles at different orientations (dR(θ)); see glossary in Box 1 for further information). Measurements of these statistical functions are not properly described by the PRW model, not even qualitatively²⁷. Rather, HT-1080 cells in a 3D matrix exhibit an exponential-like distribution of cell displacements instead of the predicted Gaussian distribution²⁷. We further demonstrated that individual cells, both on 2D substrates and inside 3D matrices, display highly variable motility patterns, which requires the incorporation of cell heterogeneity (i.e. cell-to-cell variations) in cell motility models. The incorporation of cell heterogeneity into the PRW model is sufficient to fully explain the exponential distribution of cell displacements on 2D surfaces²⁷.

However, we also showed that, even when including cell-to-cell variations, the PRW model did not properly describe cell motility in 3D ECM matrices, not even qualitatively. Based on the morphology of 3D trajectories, the angular displacements of cell movements, and the velocity profiles over different orientations, we found that cell movements in 3D matrices were highly anisotropic instead of isotropic as presumed by the PRW model. As a result, we introduced a new model, the anisotropic persistent random walk model (APRW), to describe anisotropic cell movements in 3D matrices. We showed that the APRW model qualitatively and quantitatively describes 3D cell motility for a wide range of collagen densities²⁷.

Overview of the procedure

In this protocol, we describe in detail the general procedure used to analyze the migration of eukaryotic cells in 3D settings. We illustrate this procedure by analyzing the motility of HT-1080 human fibrosarcoma cells moving in a dense 3D collagen matrix, in the presence and the absence of the actin-disassembly drug latrunculin B (Fig. 1). We also measured motilities of four additional cell types: human breast cancer MDA-MB-231 cells, human T

lymphocyte Jurkat cells, human ovarian cancer HEY cells, and human prostate cancer DU-145 cells, under different conditions. Model fitting results using the PRW model and the APRW model are shown in Table 1 and Table 2. The detailed procedures to prepare and track cells on 2D substrates and in 3D matrices, in order to produce the data for use in this protocol, can be found in Supplementary Methods.

First, an evaluation of time-invariance of motility processes is performed (Step 2). This is important, as quantities such as MSD, ACF, and angular distributions of cell movements require cell motility to be time-invariant to be meaningful and be properly computed, i.e. micro-environmental cues cannot change during the experiments. Since no sign of transient motility behavior is observed, the following statistical functions are computed from the time-dependent coordinates of the cells (i.e. the cell trajectories) (Steps 3–7): MSD, ACF, PDF-dR, PDF-d θ and dR(θ). We examine these different statistical functions to provide the most rigorous analysis of cell motility. To be noted, even though there are many conditions not tested by our study, motility statistical profiling (Steps 3–7) can be used to characterize cell migration for any cell type. To test whether the PRW model fits experimentally observed cell trajectories, individual MSD profiles are first fit with the PRW model to obtain the corresponding persistence (P) and speed (S) for each tracked cell (Step 8). PRW simulations of trajectories using these fitted P and S parameters are then performed (Step 9). To determine whether the PRW model accurately describes experimental cell trajectories, the same set of statistical tests are then performed (MSD, ACF, PDF-dR, PDF-d θ and dR(θ)) on simulated trajectories and compared with the ones directly derived from experimental cell trajectories (Step 10).

A similar procedure is used to determine whether the APRW model properly describes cell trajectories. First, individual MSD profiles are fit with the APRW model to obtain the APRW model parameters for each tracked cell (Steps 11–13). These parameters are then used to simulate cell trajectories using the APRW model²⁷ (Step 14). If the APRW model correctly describes experimental cell trajectories, the computer-simulated cell trajectories should show similar morphology. Further, statistical profiling of computer-generated cell trajectories (MSD, ACF, PDF-dR, PDF-d θ and dR(θ); Step 15) shows both qualitative and quantitative agreement with those obtained from observed cell trajectories (Step 16). Calculation of the root mean squared error (RMSE) and/or R-squared value is used for quantitative evaluation of the goodness of fits with the two different models (Step 17). If the APRW model properly describes cell trajectories and their associated statistical profiles, the persistence time (P_p , P_{np}) and diffusivity (D_p , D_{np}) along both the primary and non-primary directions of migration and the anisotropic index of migration (Φ) can be calculated to quantitatively characterize observed cell motility patterns. Steps for the computation of these parameters are described in detail in the PROCEDURE section.

Application of methods and limitations

The APRW model was developed to describe and characterize the polarized cell motility observed in 3D cell migration in the absence of symmetry-breaking gradients such as chemical and electric gradients²⁷. We have tested this analysis for a wide range of conditions, including different ECM densities²⁷ and molecular perturbations using actin

cytoskeleton disrupting drug Latrunculin B and Myosin II inhibitor Blebbistatin. The APRW model also describes the non-polarized migration of cells on 2D substrates and in this case a low value of the anisotropic index (an index of anisotropic cell movements) is expected²⁷. The APRW model may not be applied to the study of chemotaxis and durotaxis, since in this type of study cells are subjected to symmetry-breaking gradients. However, statistical functions of the cell trajectories extracted through the protocol (Steps 3–7) can provide essential information about cell motility even in these scenarios. One inherent limitation of the APRW model is that it can only apply to 2D and 3D trajectories but not pure 1D trajectories. However, 1D-like cell trajectories under a 2D observation system is still applicable. For example, the APRW analysis will work for the study of cell migration inside micro-channels^{28, 29} and on linear adhesive patterns³⁰ in which cells are only allowed to migrate in a specific direction. Applying the APRW model to this type of study, the information extracted from primary migration direction corresponds to the migration along the direction in which the cells are not confined. The information obtained along the non-primary direction of migration is less relevant. In general, obtaining the statistical profiles (MSD, ACF, PDF-dR, etc.) of cell trajectories (Steps 3–7) is applicable to all kinds of trajectory data to provide insightful information from cell trajectories.

The analysis in this manuscript is performed using 2D trajectories of cells migrating in 3D collagen I matrices. It is to be noted that obtaining 3D cell trajectories will certainly provide more accurate description of cell migration. However, since a vast majority of studies measure cell trajectories in 2D (even for migration inside 3D matrices)^{31, 32}, we have shown that the 2D APRW model can fit well the 3D motility profiles and can be used to compare migration parameters across different conditions²⁷. However, absolute value of migration parameters in 3D system may be underestimated by the projected 2D trajectory.

One basic assumption for applying the PRW and APRW models is that cell motility is time-invariant. Model-fitted results are not valid if cell speed shows large transients, regardless of the goodness of the fits. For example, studies of cell motility under a time-changing chemotactic gradient or osmotic shock are not suitable to apply this analysis. The quality and reliability of cell velocity analysis is also highly dependent on image acquisition procedures. If one uses a motorized stage during image acquisition, the vibrations or drift of that stage will translate into apparent cell movements²⁷. If stage vibration is negligible, then the tracked cell trajectories can be directly used for the analysis without correction. However, if stage vibration is significant, then a process that can eliminate or greatly reduce the effect of a moving stage should be incorporated. A common correction method consists in tracking stagnant objects (fiducial markers) in the field of view and use the relative coordinates of these objects to correct the computed coordinates of the cells.

Comparison with other methods

One straightforward and commonly used method to characterize cell motility is cell speed (displacement/time lag)^{33, 34}. However, cell motility is a stochastic process and hence cell speed depends on the choice of time lag. Using cell speed to characterize cell migration may lead to meaningless comparisons among different experimental results collected at different time lags.

Furthermore, the spatial resolution for cell positions also affects the computation of cell speed from trajectories. The positioning noise can cause an overestimation of cell speed^{35, 36}. This may potentially cause misinterpretation of trajectories. In our APRW model analysis, we not only consider the effect of positioning noise, but also extract parameters that are independent of time lag. Hence, the analysis of cell motility using the APRW model is more rigorous and comprehensive than a direct comparison of cell speed for different cellular conditions.

MATERIALS

EQUIPMENT

- Cell trajectories data (see Supplementary Methods)
- A computer running Microsoft Windows with at least 2 GB of RAM.
- The MATLAB software (see Supplementary Software).
- Windows Office software (Excel).

EQUIPMENT SETUP

- Download scripts from Supplementary Software. Open MATLAB. Set path file to the locations of scripts in MATLAB. In order to do so, open the MATLAB, then click on “Set Path” on top the panel of the main windows. Click on “add with subfolders”, and select the locations of downloaded scripts. Once the path file added to the system, click “save” and leave that window by clicking “close”.

PROCEDURE

Preparation of cell trajectory data ● TIMING 5–10 min

- 1| Following cell tracking experiment (see Supplementary Methods), export cell trajectories data from the cell tracking software into an excel file. The column in the excel file must be arranged in the following order; cell ID, time frame, x and y coordinates of the cell (typically the cell centroid) in consistent units of length (typically micrometers). An example of excel file can be found in Supplementary Data. Note that, by default, the software will identify the minimum frame length ($N_{\min}$) of all cell trajectories and the software will only adopt the first $N_{\min}$ frames of a trajectory for further analysis.

? TROUBLESHOOTING

Characterization of statistical profiles of cell trajectories ● TIMING 1–3 min

- 2| Evaluate cell velocity over time. Compute cell velocity at different time frames to examine whether the cell velocity is a time-invariant process. To obtain the velocity profile over time, run the code *get_voft.m*, as follows. Open MATLAB program. Run *get_voft.m* either by typing *get_voft* in the command window or opening *get_voft.m* in the MATLAB code editor and press run button on the top panel. Select the location of the cell trajectories file through the automated pop-out

window. Once computation is complete, another window will appear where the user will specify the filename and destination to output results to an excel file. In the exported file, the ensemble-averaged cell velocity at different time is saved under the sheet name “mean velocity over time”.

- 3| Compute the mean squared displacement (MSD) from cell trajectories. The MSD of individual trajectories (i.e. the time-dependent x and y coordinates of each cell centroid) at different time lags is computed from the following equation:

$$MSD(\tau) = \langle (x(t+\tau) - x(t))^2 + (y(t+\tau) - y(t))^2 \rangle,$$

where $\tau = n \cdot t$ and $n = 1, 2, \dots, N_{min} - 1$, and t is the time step size in trajectories acquisition. Use the code *get_MSD.m* to obtain MSDs from cell trajectories. Select the cell trajectories file, specify trajectory time step size (t), and assign the output file for MSD results following the pop-up window. A figure will display the results of the ensemble-averaged MSD profile among all cell trajectories. In the exported MSD file, both MSDs of individual cells and their ensemble-averaged value are saved under the sheet name “individual cell MSD” and “average MSD”.

? TROUBLESHOOTING

- 4| Compute the auto-correlation function (ACF) of cell velocities. The ACF of cell velocities is obtained from the following equation:

$$ACF(\tau) = \langle dx(t)dx(t+\tau) + dy(t)dy(t+\tau) \rangle,$$

where

$$dx(t) = x(t+dt) - x(t);$$

$$dy(t) = y(t+dt) - y(t).$$

$$\tau = n \cdot dt$$

$$n = 1, 2, \dots$$

To obtain the velocity ACF, run the code *get_ACF.m*. Select the cell trajectories file, specify trajectory time step size (t), and assign the output file for ACF results following the pop-up windows. A figure will appear showing the ensemble-averaged ACF profile for all cell trajectories. By default, dt is set to t to compute dx and dy . Note that the intrinsic noise present in tracked cell positions result in the overestimation of $ACF(0)$ and the underestimation of $ACF(dt)$ by an amount that is proportional to the variance of the noise²⁷. Therefore, ACF data at $n=0$ and $n=1$ are

not included in the ACF profiles. Furthermore, the ACF is normalized by $ACF(2dt)$.

? TROUBLESHOOTING

- 5| Obtain the probability density function of cell displacement (PDF-dR). Cell displacements at a time lag of dt along the x - and y -axis were obtained using the equation described in Step 3. Absolute values of displacements along both axes are used to extract the probability density function of cell displacements. To assess the PDF-dR of cell trajectories, run the code *get_dR_PDF.m*. Select the cell trajectories file, specify time lag (dt , in units of frame) for calculating displacements and assign the output file for results of PDF-dR following the pop-up windows. A figure will appear showing the results of the ensemble-averaged PDF-dR profile among all cell trajectories.
- 6| Obtain the probability density function of angular displacements (PDF- $d\theta$). Angles between two consequent steps of movements were calculated using the following formula;

$$d\theta(t) = \arccos\left(\frac{dR(t) \cdot dR(t+dt)}{|dR(t)| |dR(t+dt)|}\right)$$

$$\text{where } dR(t) = (dx(t), dy(t))$$

$$dx(t) = x(t+dt) - x(t);$$

$$dy(t) = y(t+dt) - y(t).$$

To assess the PDF- $d\theta$ of cell trajectories, run the code *get_dtheta_PDF.m*. Select the cell trajectories file and assign the output file for results of PDF- $d\theta$ following the pop-up windows. A figure will appear showing the results of the ensemble-averaged PDF- $d\theta$ profile among all cell trajectories. By default, dt is set as t , $10 \cdot t$ and $40 \cdot t$ and the number of bins between 0° to 180° to calculate the distribution of $d\theta$ is set at 6.

- 7| Obtain the velocity magnitude polarization profile ($dR(\theta)$). First, determine the primary migration direction for each of migratory trajectories of individual cells (R) by applying a singular value decomposition (SVD) to the velocity matrix of individual cells $(\overline{M})^{27}$, i.e.

$$M = U \lambda V^*$$

Here U is the matrix of eigenvectors of the product MM^* , V^* is the matrix of eigenvectors of the product M^*M , λ are the singular values of the matrix M , and $*$ denotes the transposed matrix. The first and the second eigenvectors of V^* correspond to the primary migration axis ($\vec{p}$) and non-primary migration axis ($\vec{np}$), respectively.

Re-aligned coordinates of individual cell trajectories (R_{rot}) along the primary migration direction and non-primary migration direction are obtained by $R_{rot} = R \cdot V^*$, so that the movements along the $\vec{p}$ and $\vec{np}$ axes of R become the x and y axes of R_{rot} . The average cell velocity magnitude along different orientations is computed from the re-aligned coordinates. Cell velocity is further normalized by the mean velocity.

To obtain the velocity magnitude polarization profile, use the code *get_dR_polarity.m*. Select the cell trajectories file, specify time lag (dt), and assign the output file for results of $dR(\theta)$ following the pop-up windows. A figure will appear showing the results of the ensemble-averaged $dR(\theta)$ profile among all cell trajectories. By default, dt is set to t , the time step size, to calculate the velocity matrix of cell ($\overline{M}$) and the number of bins between 0° to 180° to calculate the distribution of $d\theta$ is set at 20.

Fitting of cell trajectories to the PRW model ● TIMING 1–3 min

- 8| Obtain the PRW fitting parameters from individual cell trajectories. The MSD_{2D} of cell trajectories is fitted with the PRW 2D model using the equation below,

$$MSD(\tau) = 2S^2P(\tau - P(1 - e^{-\tau/P}) + 4\sigma^2),$$

where S is the cell speed, P is its persistence time, and $4\sigma^2$ is the noise (error) in the position of the cell.

We have developed the following procedure to perform the fitting of the PRW model. A weighting function (W) is designed to compensate for the loss of resolution in the computation of MSDs for increasing time lag and is given by

$$W(n) = MSD(n\Delta t)^1 \cdot (2n^2 + 1)^2 / (3n(N_{\min} - n + 1))^2$$

Furthermore, only the first half of MSD data is used to fit MSD since the time lags after half of observation time period are highly dependent on each other. A non-linear least square fit method is used to perform fitting.

To obtain PRW model-fitting results, use the code *fit_PRW.m*. Select the cell trajectories file, specify trajectory time step size, and assign output file for PRW model fitting results following the pop-up windows. In the output file, there are fitted values of persistent (P) and speed (S), R-squared and root mean squared value (RMSE) for individual cell trajectories.

Statistical profiling of cell motility when fitted with the PRW model ● TIMING 1–5 min

- 9| Obtain computer-simulated cell trajectories based on fitted PRW model parameters. Use the code *sim_PRW.m*. Select the file for PRW fitting results from Step 8, specify time step size for simulated trajectories, and assign output file for simulated trajectories following the pop-up windows. Details about simulations can be found in Wu *et al.*²⁷. For each individual fitted set of P and S values, repetitive simulations of trajectories are performed for improved resolution in the estimation of the statistical functions. By default, 20 repeated simulations are used for a given individual cell trajectory.

? TROUBLESHOOTING

- 10| Obtain the statistical characteristics from the computer-simulated PRW trajectories by repeating Steps 2–6, but selecting the file of simulated PRW trajectories from Step 9.

? TROUBLESHOOTING

Fitting of cell trajectories to the APRW model ● TIMING 1–3 min

- 11| Obtain the APRW fitting parameters from individual cell trajectories. Identify primary migration direction and non-primary migration direction of individual cell trajectories through SVD analysis of the cell velocities, as described in Step 7. Then realign cell trajectories to their primary and non-primary directions (i.e. $R_{rot}(t) = (x_{rot}(t), y_{rot}(t))$). Note that the primary migration direction is orthogonal to the non-primary migration direction.
- 12| Compute MSDs for each cell trajectory. MSDs along the primary and non-primary migration directions are calculated using the following equations:

$$MSD_p(\tau) = \langle (x_{rot}(t+\tau) - x_{rot}(t))^2 \rangle$$

$$MSD_{np}(\tau) = \langle (y_{rot}(t+\tau) - y_{rot}(t))^2 \rangle$$

- 13| Fit MSDs along the primary and non-primary directions using a 1D PRW model. Fit MSDs using the following equations:

$$MSD_p(\tau) = S_p^2 P_p (\tau - P_p (1 - e^{-\tau/P_p}) + 2\sigma_p^2);$$

$$MSD_{np}(\tau) = S_{np}^2 P_{np} (\tau - P_{np} (1 - e^{-\tau/P_{np}}) + 2\sigma_{np}^2).$$

To obtain APRW model-fitting results (Steps 11–13), use the code *fit_APRW.m*. Select the cell trajectories file, specify trajectory time step size, and assign the output file for APRW model fitting results following the pop-up windows. In the

output file, there are fitted values of persistent and speed, R-squared and root mean squared value (RMSE) for the primary and non-primary migration directions of individual cell trajectories.

Statistical profiling of cell motility when fitted with the APRW model ● TIMING 1–5 min

- 14| Obtain computer-simulated cell trajectories based on fitted APRW model parameters by using the code *sim_APRW.m*. Select the file for APRW fitting results from Step 13, specify time step size for simulated trajectories, and assign output file for simulated trajectories following the pop-up windows. Detailed simulation process can be found in Wu *et al.*²⁷. For fitted parameters of each trajectory using the APRW model, several simulations of trajectories are performed for improved resolution in the estimation of statistical functions (ACF, etc.). By default, 20 simulations are used for each individual trajectory.

? TROUBLESHOOTING

- 15| Obtain the statistical characteristics profiles for the simulated APRW trajectories by repeating Steps 2–6, but select the file of simulated APRW trajectories from Step 14.

? TROUBLESHOOTING

Evaluation of overall goodness of fits for the PRW and APRW models ● TIMING 5–10 min

- 16| Evaluate simulated trajectories and compare them with experimental trajectories. Run the code *show_traj.m* and first select trajectory file for experimental data following the pop-up windows. A figure will appear showing the experimental cell trajectories plot. Repeat the process to show simulated trajectories plot for both PRW and APRW models selecting trajectory files created in Steps 9 and 14, respectively.
- 17| Evaluate the goodness of model fits for the PRW and APRW models. Calculate RMSE and coefficient of determination (R^2) between statistical profiles predicted by models and measured from experimental cell trajectories.

The RMSE is calculated through the following formula:

$$RMSE = \sqrt{\langle (y_i - f_i)^2 \rangle}.$$

The coefficient of determination (R^2) is calculated using

$$R^2 = 1 - \frac{\sum (y_i - f_i)^2}{\sum (y_i - \langle y_i \rangle)^2}$$

These evaluations can apply to all aspects of motility statistical functions, including the ACF, PDF, etc.

● TIMING

Step 1: Preparation of trajectory data: ~5–10 min

Steps 2–7: Characterization of statistical profiles of cell trajectories: ~1–3 min

Step 8: Fitting of cell trajectories to the PRW model: ~1–3 min

Steps 9–10: Statistical profiling of cell motility when fitted with the PRW model: ~1–5 min

Steps 11–13: Fitting of cell trajectories to the APRW model: ~1–3 min

Steps 14–15: Statistical profiling of cell motility when fitted with the APRW model: ~1–5 min

Steps 16–17: Evaluation of overall goodness of fits for the PRW and APRW models: ~5–10 min

Anticipated Results

Figure 1 shows typical trajectories of 25 randomly chosen control (Fig. 1A) and latrunculin B-treated HT-1080 cells (Fig. 1B). Trajectories were artificially equally positioned on a grid for ease of visualization. Latrunculin B binds actin monomers, which prevents their re-incorporation into filaments, promoting rapid actin filament disassembly in cells. As expected given the critical role of actin filament assembly in cell migration, latrunculin B-treated cells migrated less than control cells as directly apparent from visual inspection of cell trajectories. Accordingly, the mean-squared displacements (MSDs) of latrunculin B-treated cells were lower than that of control cells (Fig. 1C). Latrunculin B-treated cells also showed lower occurrence of large displacements from the probability density function of cellular displacements (PDF-dR) at a time lag of 20 min (Fig. 1D). Control HT-1080 cells displayed slower decay in their autocorrelation function of cellular movements than latrunculin B-treated cells (Fig. 1E), suggesting that latrunculin-B treatment affects the persistence of HT-1080 cells. HT-1080 cells with and without latrunculin-B treatment also displayed distinct probability density functions of angular displacements (Fig. 1, F and G). The angular displacement distribution of latrunculin B-treated cells (Fig. 1G) was flatter than that of control cells (Fig. 1F), which indicates that treated cells move in random directions. However, both conditions showed similar velocity profiles at different orientations relative to the primary migration axis ($dR(\theta)$) (Fig. 1H), suggesting that cell movements follow the similar degree of anisotropy in both conditions. Figure 2 shows computer-simulated trajectories of cells obtained using parameters generated by the APRW (Fig. 3, A and B) and PRW (Fig 3, C and D) models. The trajectories obtained using these two models are qualitatively similar to the cell trajectories observed experimentally (Fig. 1, A and B).

Figure 3 compares the statistical functions generated by the APRW and PRW models with the experimental data. Quantification of the MSDs of the simulated trajectories generated by the APRW (green line) and PRW (light blue line) models match closely the experimental MSDs of control and latrunculin B-treated cells as shown in Fig. 3, A and B. Figure 3 also

compares the probability density function of cell displacements (Fig. 3C), the autocorrelation function of cell velocities (Fig. 3D), the angular distribution of cells (Fig. 3, E and F), and the orientation of cell velocities relative to the primary axis (Fig. 3G) generated by the APRW model for control and latrunculin B-treated cells. Finally, Figure 3 compares the probability density function of cell displacements (Fig. 3H), the autocorrelation function of cell velocities (Fig. 3I), and the angular distribution of cells (Fig. 3, J and K), and the orientation of cell velocities relative to the primary axis (Fig. 3L) generated by the PRW model for control and latrunculin B-treated cells. The APRW model describes the experimental data more accurately than the PRW model (Fig. 3, C–L).

Figure 4 shows the various parameters that can be extracted from the APRW fits of the experimental data. Analysis of the mean persistent time of cell migration in the primary (Fig. 4, A) and non-primary (Fig. 4, B) axes of cell migration shows that control cells are more persistent than latrunculin B-treated cells. Similarly, the diffusivities of control cells are larger than for latrunculin B-treated cells (Fig. 4, C and D) along both the primary axis and the non-primary axis of cell migration. The total diffusivity, defined as the sum of diffusivities along the primary and non-primary axes, gives an estimate of the speed of cells at long time scales (Fig. 4, E). The anisotropic index defined as the ratio of diffusivity in the primary axis over diffusivity in the secondary axis describes the degree of polarization of cell movements. As expected, the trajectories of control cells are more polarized than the latrunculin B-treated cells (Fig. 4, F).

Table 2 summarizes the goodness of fits using the PRW model and the APRW model for 11 different conditions. Statistical profiles (MSD, ACF, PDF-dR_{2min}, PDF-dR_{20min}, PDF-dθ, and dR(θ)) predicted by the two models were compared with experimental results and R² values were computed to evaluate the goodness of fits. The APRW model describes well all tested statistical profiles (R² > 0.5) for 8 conditions (including 2D, 3D and 3 different types of cells). The APRW model accurately describes all statistical profiles except the ACF for HEY cell migration (R² = -0.52) on 2D substrates, the dR(θ) for Jurkat T cell migration (R² = -0.35) on 2D substrates, and the PDF-dθ (R² = -0.08) for DU-145 cell migration in 2mg/ml collagen matrices. On the other hand, the traditional PRW model better describes cell migration on 2D substrates. Moreover, neither the PRW nor the APRW model accurately describe 2D motility of HEY cells and JURKAT T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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BOX 1**GLOSSARY****Cell trajectory ($R(t)$)**

positions of a cell at different time points over the course of time t of observation. Usually, the time step between successive cell positions is a constant expressed in units of minutes.

Re-aligned cell trajectory ($R_{rot}(t)$)

cell trajectory for which its x-axis is aligned (rotated) onto its primary migration axis. The primary migration axis is identified using the singular value decomposition (SVD) analysis of velocities of the cell.

Mean squared displacement (MSD)

average displacements of a cell evaluated at different time lags. The time lag (also called time span or time scale) is a multiple of the time step. The MSD is the most common measure of random cell movements.

Auto-correlation function of cell velocities (ACF)

ACF measures the correlation of cell velocities at different time lags (dt). A higher ACF value indicates that cell movements are more persistent. If a cell follows random walk statistics, then the ACF is approximately zero for $dt > 0$. For persistent random walk statistics, the ACF decays exponentially with an increment of dt .

Probability density function of cell displacements (PDF-dR)

occurrence or probability distribution of cell displacements. For random and persistent random walks, cell displacements follow a Gaussian distribution.

Probability density function of angular displacements (PDF- $d\theta$)

occurrence of cell angular displacements (the orientation difference between consecutive cell velocities). This profile provides statistical information on how a cell decides its direction of movement. If the cell velocity is random, then the cell will have the same chance of angular displacement in any direction ($0^\circ \sim 180^\circ$). If cell velocities are correlated in time ($ACF > 0$), then there is an elevated chance of angular displacements around an angle of 0° . If cell movements are restricted to one dimension or if they are spatially anisotropic, then there is an elevated chance of angular displacements along the 0° and 180° directions.

Velocity magnitude polarization profile ($dR(\theta)$)

average magnitude of cell speed evaluated at different orientations after re-alignment along the primary migration direction. This function reveals the degree of anisotropy of cell velocities. If the velocity is isotropic, as in the case of a true random walk or a persistent random walk, the average magnitude of cell speed is equally likely in all directions. If the velocity is anisotropic, as it is the case for 3D migration, the average

magnitude along the primary migration direction is significantly higher than along other directions.

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Figure 1. Characterization of cell migration inside a 3D matrix

(A and B) Trajectories of 25 randomly selected control (A) and latrunculin B-treated HT-1080 human fibrosarcoma cells (B) inside a 3D 2-mg/ml collagen I matrix. The trajectories were equally spaced on a grid to help visualization. The concentration of latrunculin B was 100 nM. (C) Corresponding mean squared displacements of control (black dots) and latrunculin B-treated cells (red dots). (D) Probability density function of cell displacements of control (black line) and latrunculin B-treated cells (red line). These distributions are displayed in a log-linear plot to highlight the fact these distributions are exponential. (E) Auto-correlation functions (ACF) of 2 min cell velocities. (F and G) Distributions of angular displacements of control (F) and latrunculin B-treated cells (G). (H) Orientation of the velocities of cell migration relative to the primary axis. This graph

indicates that the movements of cells in a 3D collagen matrix are intrinsically anisotropic, even in the absence of chemotactic gradients.

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Figure 2. Computer simulations of cell trajectories using the APRW and PRW models
 (A–D) Representative computer-generated trajectories of control and latrunculin B-treated HT-1080 human fibrosarcoma cells moving inside a 3D collagen matrix using the APRW (A and B) and PRW (C and D) models. Same conditions as in Fig. 1.

Figure 3. Statistical analysis of simulated trajectories

(A and B) Mean squared displacements of simulated trajectories of control and latrunculin B-treated cells generated by the APRW model (A, green line) and the PRW model (B, light blue line) overlaid with the experimental data. (C and D) Probability density functions of cell displacements (C, green lines) and autocorrelation function of cell velocities (D, green lines) generated by the APRW model overlaid with the experimental data. (E and F) Distributions of angular displacements of control (E) and latrunculin B-treated cells (F) generated using the APRW model, which can be compared to the experimental data in

Figure 1 F and G. (G and H) Probability density functions of cell displacements (G, light blue lines) and autocorrelation function of cell velocities (H, light blue lines) generated by the PRW model overlaid with the experimental data. (I and J) Distributions of angular displacements of control and latrunculin B-treated cells generated by the PRW model, which can be compared to the experimental data in Figure 1 F and G. (K and L) Orientation of velocities of cell migration obtained from trajectories generated by the APRW model (K, green lines) and the PRW model (L, light blue lines) overlaid with the experimental data. Same conditions as in Fig. 1.

Figure 4. 3D cell migration parameters obtained via the APRW and PRW models

(A and B) Mean values of persistence time along the primary (A) and non-primary axis of cell migration (B), P_p and P_{np} . (C and D) Diffusivity of cell migration (D_p or D_s) calculated

as $\frac{S^2 P}{4}$ along the primary (C) and the non-primary axis (D). (E) Total diffusivity of cell

migration obtained from APRW fits calculated as $D_{\text{total}} = \frac{S_p^2 P_p + S_{np}^2 P_{np}}{4}$. (F) The

anisotropic index (ϕ) of cell migration calculated as the ratio $\frac{D_p}{D_s} = \frac{S_p^2 P_p}{S_{np}^2 P_{np}}$. (G and H) Mean persistence time of cell migration obtained from PRW fits (G) and diffusivities of cells

calculated as $D = \frac{S^2 P}{2}$ (H). Error bars represent standard error of the mean (SEM) and One-way ANOVA was used to compute statistical significance, which is shown in the graphs using Michelin grade scale *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

Table 1

Summary of the goodness of fits (R^2) using the PRW model and the APRW model for 11 different cell conditions.

Cell name	Condition	Goodness of fit (R^2)-PRW model						Goodness of fit (R^2)-APRW model						Recommended model
		MSD	ACF ^d	P(dR _{2min})	P(dR _{20min})	dR(θ)	P(θ) ^e	MSD	ACF ^d	P(dR _{2min})	P(dR _{20min})	dR(θ)	P(θ) ^e	
HT1080	2D ^a	1.00	0.99	0.78	0.97	0.75	0.80	1.00	0.98	0.81	0.98	0.67	0.85	PRW, APRW
HT1080	3D (2mg/ml) ^b	1.00	0.94	0.57	0.69	0.38	0.74	1.00	0.95	0.78	0.91	0.98	0.93	APRW
HT1080	3D (2mg/ml) ^b , Latrunculin B, 100nM	1.00	0.75	0.76	0.73	0.26	0.80	1.00	0.76	0.96	0.94	0.99	0.92	APRW
HT1080	3D (2mg/ml) ^b , blebbistatin, 15μM	1.00	0.87	0.88	0.86	0.23	0.71	1.00	0.87	0.95	0.94	0.94	0.92	APRW
MDA-MB-231	2D ^a	1.00	0.94	0.82	0.97	0.46	0.65	1.00	0.94	0.78	0.97	0.58	0.66	APRW
MDA-MB-231	3D (2mg/ml) ^b	1.00	0.62	0.50	0.87	0.35	0.60	1.00	0.60	0.80	0.97	0.97	0.77	APRW
JURKAT T-cells	2D ^c	1.00	0.89	0.82	0.87	0.11	0.65	1.00	0.84	0.82	0.84	-0.35	0.64	PRW
JURKAT T-cells	3D (0.5mg/ml) ^b	1.00	0.57 [*]	0.92	0.46	0.66	0.51	1.00	0.53 [*]	0.93	0.50	0.80	0.52	APRW
JURKAT T-cells	3D (1mg/ml) ^b	0.91	0.58 [*]	0.91	0.83	0.40	0.70	0.90	0.50 [*]	0.92	0.77	0.72	0.77	APRW
DU-145	3D (2mg/ml) ^b	0.89	0.03 [*]	0.87	0.87	0.29	0.14	0.83	0.01 [*]	0.96	0.97	0.93	-0.08	APRW
HEY	2D ^a	0.99	-1.49	0.66	0.95	0.38	0.40	1.00	-0.52	0.68	0.98	0.68	0.55	APRW

R^2 less than 0.5 is highlighted in red;

^{*} Measured persistent time is less than 3 t. In this case, resolution of ACF plot is highly limited and R^2 value is irrelevant.

^a Collagen coated surface;

^b parenthesis indicates collagen density;

^c Fibronectin coated surface;

^d Only first 50 time lag points of ACF were used to compute R^2 value;

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R^2 for $P(d\theta)$ is based on $P(d\theta)$ and its cumulative distribution function obtained from time lag of t , $10t$, $20t$, $30t$ and $40t$.

Abbreviations:

MSD: Mean-Squared Displacement; ACF: Autocorrelation function of cell velocity; $P(dR)$: Probability density function of cell displacements; $dR(0)$: Velocity magnitude polarization profile; $P(0)$: Probably density function of angular displacement.

Table 2
Summary of the motility parameters obtained from both PRW and APRW model for 11 different cell conditions.

Cell name	Condition	PRW model				APRW model				Φ
		N	P (min)	D ($\mu\text{m}^2/\text{min}$)	P _p (min)	P _{np} (min)	D _p ($\mu\text{m}^2/\text{min}$)	D _{np} ($\mu\text{m}^2/\text{min}$)	D _{total} ($\mu\text{m}^2/\text{min}$)	
HT1080	2D ^a	64	8.8 ± 5.9	6.61 ± 5.02	11.1 ± 11.0	7.2 ± 39.5	4.83 ± 4.93	2.29 ± 1.96	7.11 ± 5.89	3.6 ± 5.3
HT1080	3D (2mg/ml) ^b	69	14.5 ± 16.6	2.02 ± 3.23	17.4 ± 21.0	7.1 ± 24.2	1.85 ± 3.03	0.27 ± 0.51	2.12 ± 3.40	13.6 ± 18.0
HT1080	3D (2mg/ml) ^b , Latrunculin B, 100nM	100	14.3 ± 18.6	0.39 ± 0.64	17.1 ± 23.7	8.0 ± 5.3	0.34 ± 0.66	0.07 ± 0.10	0.41 ± 0.69	8.5 ± 25.0
HT1080	3D (2mg/ml) ^b , blebbistatin, 15μM	100	18.2 ± 23.2	1.00 ± 2.04	21.2 ± 28.4	7.6 ± 16.0	0.93 ± 2.00	0.12 ± 0.23	1.05 ± 2.14	20.8 ± 45.9
MDA-MB-231	2D ^a	38	10.5 ± 23.4	9.49 ± 13.41	13.0 ± 28.8	5.6 ± 112.3	7.38 ± 14.04	2.77 ± 3.40	10.15 ± 14.94	7.2 ± 26.2
MDA-MB-231	3D (2mg/ml) ^b	76	6.4 ± 14.2	0.35 ± 0.51	7.7 ± 17.7	3.4 ± 4.3	0.30 ± 0.54	0.07 ± 0.06	0.37 ± 0.55	5.3 ± 8.1
JURKAT T-cells	2D ^c	87	23.0 ± 47.8	83.72 ± 111.19	44.1 ± 118.8	11.0 ± 1696.3	100.40 ± 212.07	17.87 ± 20.30	118.27 ± 219.25	47.2 ± 387.1
JURKAT T-cells	3D (0.5mg/ml) ^b	30	4.2 ± 6.5	1.76 ± 1.95	6.3 ± 11.6	3.5 ± 13.3	1.30 ± 1.66	0.58 ± 0.74	1.88 ± 2.14	2.8 ± 2.4
JURKAT T-cells	3D (1mg/ml) ^b	46	1.9 ± 4.1	0.83 ± 1.60	2.7 ± 5.2	0.8 ± 8.0	0.55 ± 1.00	0.28 ± 0.66	0.84 ± 1.60	3.7 ± 3.8
DU-145	3D (2mg/ml) ^b	14	0.4 ± 0.5	0.10 ± 0.09	1.2 ± 2.6	0.7 ± 0.5	0.07 ± 0.07	0.03 ± 0.02	0.10 ± 0.09	2.2 ± 1.1
HEY	2D ^a	34	13.8 ± 12.1	1.30 ± 1.69	21.5 ± 23.8	7.9 ± 16.5	1.41 ± 2.06	0.19 ± 0.24	1.60 ± 2.11	14.2 ± 18.5

Values show the motility parameters (mean ± std) from PRW and APRW model fitting;

- ^aCollagen coated surface;
- ^bparenthesis indicates collagen density;
- ^cFibronectin coated surface;

Abbreviations:
N : Sample size; P: Persistent time; D: Cell diffusivity; P_p, P_{np}: Persistent time in primary and non-primary migration direction; D_p, D_{np}: Cell diffusivity in primary and non-primary migration direction;
D_{total}: Total cell diffusivity (D_p+D_{np}). Φ : Anisotropic index ($\equiv D_p/D_{np}$)

Troubleshooting table.

Step	Problem	Possible reason	Solution
1	Error message: index out of boundary	Empty excel cells present in the trajectories file	Correct empty cells in the trajectories excel file and make sure there are no empty cells present in the trajectory data.
3	Error message: deletesheet function is not defined	The software may not compatible in MAC OS.	Use Windows OS to run the program.
4	Discontinuous ACF function.	Outlier cells/Immobile cells	Delete outlier cells or use linear scale to show the results. If most cells are immobile, do not use ACF to evaluate the goodness of PRW and APRW fits.
9,14	Taking too long to simulate the results	Selected the wrong file	Stop the process (ctrl+c). Rerun the program and select the correct file
9,14	Error message: Error using xlswrite error code: 0x800A03EC.	Simulated trajectory data exceeds maximum row limits in Microsoft Excel	Reduce the number of simulations repeats.
4,10,15	ACF shows a broken profile	Persistence in cell velocity is low, hence the memory in cell velocity is approximately zero hence the zero in normalized ACF curve spikes.	If the simulation results show similar profiles as in experimental results. Check the fitted persistence value, and if the fitted value is within the range of less than 3 frames. This will confirm the broken curve of ACF results from a low persistence instead of an error.