Identifying Transport Behavior of Single-Molecule Trajectories

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ABSTRACT Models of biological diffusion-reaction systems require accurate classification of the underlying diffusive dynamics (e.g., Fickian, subdiffusive, or superdiffusive). We use a renormalization group operator to identify the anomalous (non-Fickian) diffusion behavior from a short trajectory of a single molecule. The method provides quantitative information about the underlying stochastic process, including its anomalous scaling exponent. The classification algorithm is first validated on simulated trajectories of known scaling. Then it is applied to experimental trajectories of microspheres diffusing in cytoplasm, revealing heterogeneous diffusive dynamics. The simplicity and robustness of this classification algorithm makes it an effective tool for analysis of rare stochastic events that occur in complex biological systems.

INTRODUCTION

Stochastic fluctuations arise in biological systems at all length and timescales. An example is the diffusion of biomolecules, driven by thermal fluctuations, providing transport for biochemical processes. A familiar process that can generate such behavior is Brownian motion, although in biological systems the explicit assumption that particles are dilute is often violated. Diffusion processes that do not produce Brownian statistics are said to exhibit anomalous (non-Fickian) diffusion. Occurrence of anomalous diffusion has been reported in diverse phenomena, and has enriched understanding of biological systems (1).

Particle trajectories produced by single-molecule experiments are particularly suitable for characterizing biological diffusion behavior. A particle trajectory consists of \( M \) locations \( X_i = X(t_i) \), which the particle occupies at times \( t = t_i \). This trajectory is represented by a sequence of time-ordered random variables

\[
X = \{X_i\}_{i=0}^{M}.
\]

Anomalous behavior arises when a trajectory has nonzero drift, correlated increments, nonstationary increments, or some combination (2). Equivalently, Brownian motion is observed when these conditions are absent. The trajectory of a particle undergoing Brownian motion exhibits a mean-square displacement (MSD) that grows linearly with time \( t \). Anomalous diffusion typically produces an MSD, \( \langle X^2 \rangle \), which is a nonlinear function of \( t \), for example a power law

\[
\langle X^2 \rangle(t) = D_\alpha t^\alpha, \quad 0 < \alpha < 2,
\]

Here \( D_\alpha \) is the diffusion coefficient, and \( \langle \cdot \rangle \) denotes an average over an ensemble of random trajectories. For stationary processes considered in this analysis, the exponent \( \alpha \) is assumed constant throughout the time-course. The process \( X \) is called subdiffusive if the exponent \( \alpha < 1 \), and superdiffusive if exponent \( \alpha > 1 \) (3). A time-averaged MSD (TAMSD) can be used to analyze single trajectories and is defined for the \( n \)th trajectory by

\[
\overline{X_n^2}(\Delta, t) = \frac{1}{t - \Delta} \int_0^{t-\Delta} \langle [X_n(t') + \Delta] - X_n(t') \rangle^2 \text{d}t',
\]

where \( \Delta \) is a lag time (4).

Although many experiments use TAMSD as a key statistic characterizing the observed dynamics, a number of studies have demonstrated that it can be a problematic metric for classification in certain cases (2,5,6). In response, a number of algorithms have been proposed for process classification, such as \( p \)-variation (7), autocorrelation functions (8), higher order moments (9), and an ergodicity breaking parameter (10). Alternatively, approaches have been developed with a focus on experimental trajectories using empirical evidence of deviation from Brownian motion (11–13).

A recently proposed framework based on a renormalization group operator (RGO) (2,14,15) provides a promising alternative. A random trajectory \( X \) has a set of increments \( I = \{I_i\}_{i=0}^{M-1} \), with each increment computed as \( I_i = X_{i+1} - X_i \). An RGO \( R_{p,n} \) can be defined by

\[
(R_{p,n}I)_k = \sum_{k=m}^{(i+1)n-1} \frac{l_k}{m^p}, \quad p > 0, \quad n \geq 1.
\]
and a new replica trajectory $J$ is determined as $J^{p,n} = (R_{p,n}I)_i$. For a fixed $p$, a sequence $I$ is called a fixed point of the RGO if the relationship

$$J^{p,n} = I$$

holds for all $n \geq 1$, where $=d$ means equal in distribution (16). A process that is a fixed point with scaling $p$ is said to be $p$-diffusive, or $p$-self-similar, and is related to the anomalous diffusion exponent by $\alpha = 2p$ (2). A generalization is a random RGO, in which the scaling is a random variable $P$ (14). In this framework, a single experimental trajectory is a realization of a process that samples a scaling distribution $f_p(p)$. Here, we propose an algorithm to determine the anomalous diffusion exponent distribution $f_p(p)$ of a stochastic trajectory. The goal is to obtain $p$ from single, short trajectories of experimental data; other methods may be more appropriate for analytic processes (17).

**METHODS**

**Classification algorithm**

The proposed algorithm consists of the following four steps:

1. A single trajectory $X$ is transformed into the increment process $I$ and the empirical cumulative distribution function (CDF), $F_I(x)$, of $I$ is computed.

2. A constrained optimization is performed on a goodness-of-fit statistic comparing empirical CDFs. Although other options exist, good results were found by minimizing the goodness-of-fit statistic from Kuiper’s two-sample test (18),

$$g^{p,n} = \sup_x |F_I^n(x) - \inf_x |F_I^n(x) - F^{p,n}_I(x)|,$$  

where $F^{p,n}_I(x)$ is an empirical CDF of $J^{p,n} = R_{p,n}I$.

3. Optimization is repeated for many values of $n$ to obtain corresponding best-fit values of $p$ and are plotted as a histogram to obtain a distribution $f_p(p)$ for a given realization.

4. The scaling exponent is computed as $\alpha = 2p$, where $\alpha$ is the mean of the empirical distribution $f_p$, the corresponding diffusion coefficient is defined by $D_{\alpha} = \sigma^2(2\alpha d)$, where $\sigma(x)$ is the variance of $I$, $d$ is the time step, and $d$ is the spatial dimension (19).

The use of increments $I_{i+1} = X_{i+1} - X_i$ for a lag time $\Delta$ (the number of time steps) improves estimation of $D_{\alpha}$. The diffusion coefficient is then calculated as

$$D_{\alpha} = \frac{1}{M} \sum_{i=1}^{M} \frac{\sigma^2(t)}{2d(\Delta t)^{\alpha}}.$$  

This calculation also acts as an indicator of the accuracy of the calculated scaling. If an estimate of $\alpha$ is inaccurate, then $D_{\alpha}(\Delta)$ will change significantly with $\Delta$, indicating a poor fit.

A trivial extension is to perform the operation over a population of trajectories to produce an ensemble distribution. This is analogous to other methods of computing the average scaling, but has the advantage that each trajectory is treated individually, a fact particularly useful when the population contains a mixture of processes. Further refinements, such as subdividing the trajectories into subtrajectories or performing a moving window analysis, can be used to obtain information local in time.

**Stochastic processes**

Several stochastic processes are used below to validate the proposed algorithm. Brownian motion $B_t$ is a time series of Gaussian-distributed random variables with zero mean and unit variance. A Lévy flight $L_t$, can be generated by taking step increments from a stable distribution $S(\alpha_L,\delta,0)$ with $\delta = 0$ (20). Note the distribution parameter $\alpha_L$ is different from the anomalous diffusion exponent $\alpha$. The analytical MSD of $L_t$ scales like $t^{2\alpha_L}$, and is therefore $1/\alpha_L$-diffusive (21). Fractional Brownian motion (FBM) is denoted $B_t^H$ and defined with initial condition $B_0^H = 0$, zero mean $\langle B_t^H \rangle = 0$, and a two-point correlation,

$$\langle B_t^H B_s^H \rangle = \langle |t|^{2H} + |s|^{2H} - |t - s|^{2H} \rangle / 2,$$

where $H$ is the Hurst exponent. In this work, FBM is simulated using the circular method as implemented in the R package dmvBm (22,23). It can be shown that $B_t^H$ is $H$-diffusive.

**RESULTS**

**Validation**

The proposed classification scheme produces a distribution of scaling exponents for each individual trajectory, and provides an estimate of the mean and variance from the single-trajectory scaling distribution $f_p(p)$. In Fig. 1, trajectories generated by three stochastic processes described above are analyzed using the TAMSD and compared to the proposed RGO method. Ensemble averages ($N = 10^5$) are shown as a dashed line. In all cases, the use of the RGO method reduces the dispersion of the estimated scaling. Fig. 1a exhibits the $B_t$ trajectories computed with TAMSD (black) and the RGO algorithm (gray). A similar comparison for $B_t^H$, is provided in Fig. 1b for $H = 0.25$ and 0.75. For $H = 0.25$, the RGO method (light blue) converges to the ensemble mean much more slowly than TAMSD (dark blue), whereas the convergence rates of TAMSD (dark orange), and RGO (light orange) are nearly identical for $H = 0.75$. In Fig. 1c, the TAMSD analysis of $L_t$ for $\alpha_L = 0.5$ and 1.0 incorrectly predicts a scaling of $p = 0.5$ (dark purple) compared to the analytically derived scaling $p = 1/\alpha_L$ (24). The RGO method correctly characterizes the trajectories for $\alpha_L = 0.5$ (green) and 1.0 (light purple).

A subtle point is determining the limits of accurately characterizing a stochastic trajectory. Jeon et al. (25) introduced an ergodicity-breaking parameter

$$\xi_i = \frac{X_i}{\langle X^2 \rangle}$$

as a means of analysis of pre-ergodic data. Previously, we used this parameter to distinguish between stochastic processes (26). Fig. 2 exhibits the mean $\xi$ obtained with such analysis for $B_t$ and $B_t^H$. It shows weak ergodicity breaking for short ($M < 100$) trajectories, which is in agreement with the weak ergodicity breaking at short times previously observed for $B_t^H$ (27). This is also true for $B_t$, an ergodic process, suggesting there is insufficient information in a short trajectory to accurately characterize the underlying
process. Therefore, an accurate determination of the anomalous scaling exponent from single-trajectory data is undermined by the scarcity of data and pre-ergodic convergence associated with short trajectories.

Experimental data typically consist of a few trajectories that are short in time. Ten realizations of $B_H^t$ are simulated with $H = 0.25$ and $0.75$, for $M = 1000$ time steps. Their representative distributions $f_P$ are shown in Fig. 3, $a$ and $b$, for $H = 0.25$ and $H = 0.75$, respectively. In all cases, good agreement with the scaling of the underlying process is observed. The exact scaling, $\delta(x - P)$, where $\delta$ is the Dirac delta function, is replaced with a finite-width distribution that accounts for uncertainty introduced by the trajectory’s short length. An ensemble histogram of the population of 10 trajectories with $H = 0.25$ in Fig. 3 $c$ shows weak convergence to the expected mean. A mixed population of 10 trajectories from each $H$ in Fig. 3 $d$ demonstrates that the algorithm robustly...
recovers a bimodal distribution for the mixture case without additional constraints on the algorithm.

Experimental data analysis

The validated classification algorithm is used to analyze previously reported data (26). Briefly summarizing, we obtained three-dimensional particle trajectories of a fluorescent microsphere, diameter 1 μm, diffusing in a cellular extract prepared from Xenopus oocytes. Another set of trajectories was also obtained from the same extract treated with nocodazole, which is known to depolymerize microtubules. For all experiments, the total acquisition time per three-dimensional volume was 86 ms. Previously, an MSD-based analysis, using Eq. 1, classified transport as superdiffusive along microtubules in the extract case (α = 1.5 for both short and long times; and Dα = 0.014 and 0.038 in short- and long-time analyses). The units for all experimental diffusion coefficients are μm²/s, in agreement with Eq. 1. Addition of nocodazole led to subdiffusive transport at short times (α = 0.6), and classical diffusion (α = 1.0) at long times; in both cases, Dα = 0.16 (26). Fig. 4 exhibits the resulting MSDs.

Fig. 5 presents the results obtained with our classification algorithm for the extract (left column) and nocodazole (right column) cases. Distributions from single representative trajectories are shown in Fig. 5, a and b. Both cases exhibit superdiffusive and subdiffusive signatures, although they are less evident in the nocodazole case. These results indicate that the experimentally observed trajectories are characterized by process transitions. In the extract case this is not surprising, inasmuch as the dynamics of microtubule transport include rapid switching between on- and off-states as the microsphere interacts with the microtubules (28). Superdiffusive transport in the nocodazole case suggests inadequate depolymerization of the microtubules. In both cases, Dα = 0.16 (26). Fig. 5, c and d shows ensemble population distributions, with N = 38 in the extract case and N = 19 in the nocodazole case. In the extract case, subdiffusive behavior is observed, which was not indicated by the MSD analysis. Similarly, weak superdiffusive behavior is observed in the nocodazole case, suggesting incomplete depolymerization of microtubules. The large mass at P = 0 is due to occasional calculation of negative scaling exponents due to short trajectory size, but physical processes have the constraint p > 0.

Comparison to previous methods

Applying the renormalization group method using a moving window provides a useful comparison to alternative characterization methods. In this case, we compare α/2 from the classic TAMSD method, the Dev parameter derived by

![FIGURE 4 Ensemble MSD of individual TAMSDs from each experimental trajectories. (a) At short times, transport is superdiffusive in the extract case (due to active transport along microtubules) and subdiffusive in the nocodazole case. (b) At long times, transport is superdiffusive in the extract case and Fickian in the nocodazole case. To see this figure in color, go online.](image-url)

![FIGURE 5 RGO analysis of experimental data. (a and b) Representative histograms of the empirical scaling distribution fP(p) of individual trajectories from the extract and nocodazole cases, respectively. Three examples are shown with each color indicating results from a single trajectory. (c and d) Ensemble histograms for the two cases. In the extract case, subdiffusive behavior is observed, which was not indicated by the MSD analysis. Similarly, weak superdiffusive behavior is observed in the nocodazole case, suggesting incomplete depolymerization of microtubules.](image-url)
Huet et al. (11), and a velocity correlation parameter SCI derived by Bouzigues and Dahan (11,13). We compare this with the mean scaling $p$ and the evolution of the full distribution $f_P(p)$ over time. In all cases a window size of 41 time points is used.

A comparison is first made using simulated data of known scaling. The simulated trajectory is a concatenation of three distinct walks, each with 200 time steps:
1. A fractional Brownian motion with $H = 0.25$,
2. A fractional Brownian motion with $H = 0.75$, and
3. A Lévy flight with $\alpha_L = 1$.

The computed characterization is shown in Fig. 6 in the left column (Simulation). The TAMSD method works well for the fBM cases, but fails to find the correct scaling for the Lévy flight, as expected from Fig. 1. The mean $p$ is noisy, but varies around the expected scaling for each distinct process. The parameter Dev gives some qualitative information, but it is not clear how to map Dev to the actual scaling exponent. This method also fails to distinguish a Lévy flight. The correlation SCI only gives a rough qualitative sense of the change in underlying scaling, but no quantitative information. Finally, the full distribution $f_P(p)$ gives a good sense of the local fluctuations around the underlying scaling exponent, despite containing limited samples per time point due to the small window size.

These methods are also applied to one experimental trajectory shown in the right column (Extract) of Fig. 6. In this case, similar features are seen to the simulation case, although the parameters are noisier due to particle location uncertainty in experimental data. The particle appears to undergo Brownian motion in the beginning of the trajectory, switching to subdiffusive behavior at ~500–600 time points.

**CONCLUSION**

We have shown that renormalization group operators are an effective method for determining the scaling exponent of

<table>
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<tr>
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<th>Short time $D_a$</th>
<th>Long time $D_a$</th>
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<tr>
<td></td>
<td>MSD</td>
<td>RGO</td>
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<tr>
<td>Extract</td>
<td>0.014</td>
<td>0.029</td>
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<tr>
<td>Nocodazole</td>
<td>0.16</td>
<td>0.043</td>
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Units are $\mu m^2/s$.

**FIGURE 6** Moving window analysis of simulated and extract trajectories. (a1) Simulated trajectory; first 200 points are a fractional Brownian motion with $H = 0.25$, the next 200 points are an fBM with $H = 0.75$, and the last 200 points are a Lévy flight with $\alpha_L = 1$. All moving windows for the analysis of this trajectory uses a window size of 41. (a2) Experimental trajectory of microsphere diffusing in cellular extract. (b1,2) The mean value $p$ computed using the proposed algorithm. (c1,2) $a_2/2$ calculated using a moving window time-averaged mean-squared displacement analysis. (d1,2) The parameter Dev proposed by Huet et al. (11). (e1,2) The parameter SCI proposed by Bouzigues and Dahan (13). (f1,2) The full distribution $f_P(p)$ computed using the proposed algorithm. Although the distributions are sparsely populated due to the small window size, the random scaling for each process as it varies with time is clearly shown. In the extract case, the distribution suggests Brownian motion in the initial segment followed by a transition to subdiffusive behavior at ~500–600 time points. To see this figure in color, go online.
discrete stochastic processes and experimental data. The analysis suggests that whereas fluctuations in short-time windows can produce improbable sequences that scale differently from the underlying process, their average behavior is sufficient to accurately estimate the scaling exponent and to classify the process. Many biological phenomena are characterized by broad distributions for which it may be difficult (or even inappropriate) to assign a single value of the scaling exponent $\alpha$. The observed distributions are likely generated by process transitions, which reflect the presence of multiple transport mechanisms (e.g., free diffusion, varieties of cytoskeletal transport, and active transport through pores) that may act upon biomolecules during different time windows or events (29). From a regulatory perspective, diverse transport mechanisms with different scaling would provide a powerful means of control over biochemical pathways. The assortment of scalings seen in the MSD analysis of individual trajectories of diffusing chromosomal loci (30) provides further support for this conclusion. The MSD-based estimates of the scaling exponents for individual trajectories were collected into a histogram shown in Fig. 2a of Javer et al. (30). It is similar to the histogram for the extract case shown in Fig. 5c. The proposed algorithm could also be used to classify the distinct short-time diffusive behaviors observed in different subcellular regions reported by Javer et al. (30).

Nonstationary transport has been previously observed in the cytoplasm and plasma membrane of living cells (31,32), in which the distribution of anomalous diffusion exponents was a function of time and the process generating the dynamics, i.e., $f(p|t,A)$, where $A_i$ is a specific stochastic process. Here, we have assumed that the dynamics are stationary over the acquisition time, so the anomalous exponent is only a function of the generating stochastic process $f(p|A_i)$. In previous experiments, nonstationary behavior was apparent on a timescale of 100 s (31), similar to the timescale of the experiments presented here. Therefore, we are at the limit of applicability of the proposed method for stationary processes. However, classifying nonstationary processes using short trajectories is ill-posed in many cases, due to a combination of limited statistical information and an unknown rate of change in dynamics. Without sufficient accumulation of statistical information, i.e., many time points, accurately classifying such a process would be difficult at best. In addition, the proposed method does not properly characterize fundamentally nonstationary processes. Assuming sufficient data, a similar method to the one proposed here could be developed using a different RGO, similar to previous analytical work (17), but the details remain unresolved.

In summary, we developed a classification technique to analyze short trajectories of a single biomolecule based on an RGO (2), which allows computing both the anomalous scaling exponent and diffusion coefficient of a biomolecule’s motion. The RGO-based classification alleviates key impediments to accurate identification of anomalous transport, which stem from scarcity of observed biomolecule trajectories, short duration of observation, and measurement errors. After validation on simulated stochastic processes, we used the proposed method to analyze trajectories of diffusing microspheres observed in several biological environments. The RGO approach identified multiple transport processes affecting the observed particle migration, revealing behavior that had been missed in a previous analysis. The RGO method could be used to analyze single trial data from other sources in which transitions are likely to occur.

This research was supported by the Howard Hughes Medical Institute, National Institutes of Health grant No. MH079076, the Salk Innovation Award, and the Air Force Office of Scientific Research under grant No. FA9550-12-1-12-1-0185.

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