# Towards a robust criterion of anomalous diffusion

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Anomalous-diffusion, the departure of the spreading dynamics of diffusing particles from the traditional law of Brownian-motion, is a signature feature of a large number of complex soft-matter and biological systems. Anomalous-diffusion emerges due to a variety of physical mechanisms, e.g., trapping interactions or the viscoelasticity of the environment. However, sometimes systems dynamics are erroneously claimed to be anomalous, despite the fact that the true motion is Brownian—or vice versa. This ambiguity in establishing whether the dynamics as normal or anomalous can have far-reaching consequences, e.g., in predictions for reaction- or relaxation-laws. Demonstrating that a system exhibits normal- or anomalous-diffusion is highly desirable for a vast host of applications. Here, we present a criterion for anomalous-diffusion based on the method of power-spectral analysis of single trajectories. The robustness of this criterion is studied for trajectories of fractional-Brownian-motion, a ubiquitous stochastic process for the description of anomalous-diffusion, in the presence of two types of measurement errors. In particular, we find that our criterion is very robust for subdiffusion. Various tests on surrogate data in absence or presence of additional positional noise demonstrate the efficacy of this method in practical contexts. Finally, we provide a proof-of-concept based on diverse experiments exhibiting both normal and anomalous-diffusion.

## I. INTRODUCTION

The exploration of the dynamic properties of complex systems has been massively boosted by modern microscopic techniques allowing single-particle tracking (SPT) of micron- and submicron-sized tracers or even single molecules. SPT is routinely used to probe the local properties of materials and even live biological cells and tissue by passive and active microrheology [1]. SPT by now is a key tool to interrogate the structure–function relationships in biophysical applications [2, 3], and it plays a central role in uncovering thermal and energy-fuelled intracellular transport of tracer particles or single molecules in biological cells and tissues [4]. SPT is thus at the heart of the new emerging era of quantitative life sciences [2–31]. Specifically, SPT unveiled different intracellular motion patterns of virus particles [5], Cajal bodies [6], molecular motor-driven transport [7–9], the motion of telomeres [10], green fluorescent proteins [11], DNA-binding proteins [12, 13], mRNA molecules [14–16], membrane proteins [17–19], or endogenous granules [20, 21] and vesicles [29]. SPT has also revealed protein interactions [22] as well as key details of submicron tracer motion in mammalian cells [23] and in the movement ecology of larger animals [31, 32].

In contrast to pre-averaged data such as those obtained from fluorescence correlation spectroscopy (FCS) or fluorescence recovery after photobleaching (FRAP), SPT provides high resolution "unprocessed" data: As raw data of test particle trajectories, SPT offers the best possible basis for statistical analysis [33]. Indeed, a large toolbox of methods are available for analysing position time series  $X_t$ . Frequently, these data have revealed that particles exhibit an anomalous diffusion behaviour, defined by the power-law dependence  $\langle X_t^2 \rangle \simeq t^{\alpha}$ 

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of the mean squared displacement (MSD), where the angular brackets denote averaging over different realisations of recorded trajectories. The anomalous diffusion exponent  $\alpha$  is commonly used to tell whether normal (Brownian,  $\alpha = 1$ ) or anomalous diffusion ( $\alpha \neq 1$ ) is observed. Here, the regimes of sub- and superdiffusion correspond to  $0 < \alpha < 1$  and  $\alpha > 1$ , respectively. Distinguishing normal from anomalous diffusion is vital for predicting various characteristics of the systems under investigation—e.g., the diffusion-control of molecular reactions or the relaxation dynamics after manipulating the system—eventually allowing us to understand the actual physical mechanisms underlying the observations.

Fitting the scaling exponent  $\alpha$  to finite measured time series is known to be a major challenge. For instance, contamination of the true trajectories by measurement noise was shown to lead to the erroneous conclusion to observe anomalous diffusion  $(\alpha \neq 1)$  [34]. There exist methods to alleviate this problem, e.g., the mean-maximal excursion statistics [35]. Moreover, Bayesian-maximum likelihood methods [36–38], deep learning strategies [39– 43], or feature-based methods [42-45] also provide best estimates for  $\alpha$ . However, all these methods have their shortcomings. Quite severely,  $\alpha$  values (along with D values [46, 47]) retrieved from fitting the MSD  $\langle X_t^2 \rangle$  will vary from one trajectory to the next due to finite statistics within single trajectories. Indeed, trajectories from real experiments display different regimes with different scaling exponents [36, 37, 48], or due to the spatial heterogeneity of the environment [49, 50]. This leads to strong variability in the scaling exponents measured for different trajectories and renders predictions based on such fitting procedures even less accurate. Moreover, for realistic situations with  $\alpha$  values closer to the Brownian value  $\alpha = 1$  it becomes increasingly difficult to distinguish anomalous from Brownian motion [43]. This latter caveat is further exacerbated when considering the unavoidable experimental sources of error in many SPT setups, especially when based on fluorescence microscopy methods.

Likely the most notorious source of uncertainty is the static localisation error that arises from the finite number of fluorescence photons garnered during an image of the particle from which its position will be retrieved by elaborate tracking schemes: Each of these photons is emitted from a point-like source in the sample (the emitting fluorophore) and will hence be captured on a locus of the camera sensor according to the microscope's point-spread function (PSF). In other words, individual photons are stochastically recorded on the camera sensor, with a distribution of positions around the actual particle location determined by the PSF [51]. As a consequence, recording only a few photons will yield a poor estimate for the actual particle position, and the characteristic deviation is determined by the standard error. The latter scales with the inverse root of the number of photons, i.e., for a large number of photons the static localisation error can be as small as a few nanometres [52]. However, for very

large numbers of photons another perturbation becomes visible, the so-called dynamic localisation error: Recording an image to determine a particle's position takes a finite time during which particles are constantly on the move. As a result, many different positions are visited during the acquisition of a single image and only the temporal mean of these is retrieved from the acquired image as the, apparent, particle position. This dynamic localisation error effectively adds a negative offset to  $\langle X_t^2 \rangle$ whereas the static localisation error adds a positive offset [53, 54]. Both sources of error will therefore perturb the analysis of the scaling behaviour on short time scales for which experimental trajectories typically yield best statistics. Thus, determining the value of  $\alpha$  and deciding whether diffusional anomalies are present is indeed a major challenge.

The aim of this article is the analysis of a robust and easy to implement method that allows one to decide on the type and significance of an apparent anomaly, without being spoiled by localisation errors. We concentrate here on the situation when  $X_t$  belongs to a wide, experimentally-relevant class of anomalous diffusions the so-called fractional Brownian motion (FBM) [55]. Note, however, that the methodology we develop here will be amenable to generalisation to any anomalous– diffusion process. FBM is a Gaussian stochastic process characterised by a zero mean value and the covariance function

$$\langle X_{t_1} X_{t_2} \rangle = D\left(t_1^{2H} + t_2^{2H} - |t_1 - t_2|^{2H}\right),$$
 (1)

where D is a proportionality factor with physical units of length<sup>2</sup>/time<sup>2H</sup> commonly referred to as generalised diffusion coefficient and  $H \in (0, 1)$  is the traditionally used Hurst index, such that the anomalous diffusion exponent is  $\alpha = 2H$ . FBM thus describes a process that can be subdiffusive (H < 1/2), diffusive (H = 1/2), or super-diffusive (H > 1/2). From a physical point of view, FBM is well suited to model diffusion in viscoelastic media [16, 20, 56–58], but it also governs observed motion patterns in movement ecology [30], the density profiles of serotonergic brain fibres [59], or roughness in financial data [60].

As mentioned before, localisation errors are usually divided into two kind of contributions [53, 61]: the static error, due to intrinsic properties of the experimental setup, and the dynamic one, due to the finite time needed for data acquisition, i.e., the exposure time. From a mathematical point of view the former is generally treated as an independent additive noise source whereas the latter is defined via temporal integration over the finite exposure time. The effect of measurement error in SPT has been investigated mainly by focusing on the MSD [53, 62, 63]. Few results are also present in literature concerning correlation functions and power spectra [53, 54], but with a small range of applicability. Spectral analysis of stochastic processes can be very helpful in their characterisation, however the power spectrum, according to the text-book definition, is a property that relies on the

measurement time going to infinity and on a very large statistical ensemble. Both of these assumptions are typically not met when dealing with state-of-the-art SPT data, and this is why the spectral analysis of individual trajectories was only recently introduced [64]. The study of single-trajectory spectral densities has been carried out for different stochastic processes [19, 65–70] and is based on the study of the random variable [64, 65]

$$S(f,T) = \frac{1}{T} \left| \int_0^T dt e^{ift} X_t \right|^2$$
  
=  $\frac{1}{T} \int_0^T dt_1 \int_0^T dt_2 \cos\left(f(t_1 - t_2)\right) X_{t_1} X_{t_2}, \quad (2)$ 

where f is the frequency, T is the finite observation time, and  $X_t$  is an individual realisation of a given stochastic process. We will refer to this quantity as the singletrajectory power spectral density (PSD).

When the parental process  $X_t$  is Gaussian, the probability density function (PDF) of the random variable S(f,T) is, likewise, entirely defined by its first moment and variance

$$\mu(f,T) = \langle S(f,T) \rangle, \qquad (3)$$

$$\sigma^2(f,T) = \left\langle S^2(f,T) \right\rangle - \left\langle S(f,T) \right\rangle^2.$$
(4)

Note that by taking the limit  $\lim_{T\to\infty} \mu(f,T) = \mu(f)$ , we recover the standard definition of the power spectrum. Interestingly it was shown that the characteristic trend of the ensemble averaged power spectrum  $\mu(f,T)$  of several stochastic processes at high frequencies can be inferred already from a single-trajectory PSD [19, 64–69].

A very interesting quantity to study when performing single-trajectory spectral analyses is the so-called coefficient of variation of the associated PDF, P(S(f,T)),

$$\gamma(f,T) = \frac{\sigma(f,T)}{\mu(f,T)}.$$
(5)

In particular, when dealing with FBM-like motion the limiting value of  $\gamma(f,T)$  at high frequencies (or for a fixed frequency but for long T) turns out to be distinctly different in the cases of subdiffusion  $(\gamma(f, T) \sim 1)$ , normal diffusion  $(\gamma(f,T) \sim \sqrt{5}/2)$  and superdiffusion  $(\gamma(f,T) \sim \sqrt{2})$  [65]. This quantity was proposed in [65] as criterion of anomalous diffusion. Here, we go a significant step further and study how its trend towards specific limiting values is affected by the experimentally unavoidable presence of localisation errors in tracked trajectories. In particular, we show that: (i) this criterion is very robust for subdiffusion; (ii) in the case of superdiffusion the limiting value of  $\gamma(f,T)$  is affected by the static measurement error and not by the dynamic error; (iii) for normal diffusion both static and dynamic errors introduce correction terms in the limiting value of  $\gamma(f, T)$ . Knowledge of these results allows a reliable determination of the anomalous nature of measured signals.

# II. RESULTS AND DISCUSSION

As already mentioned above, for pure FBM trajectories it was shown [65] that the coefficient of variation at high frequencies reaches the limiting values: (i)  $\gamma = 1$  for subdiffusion, regardless of the value of H, (ii)  $\gamma = \sqrt{5}/2$ for normal diffusion, and (iii)  $\gamma = \sqrt{2}$  for superdiffusion, regardless of the value of H. Here, we analyse the case of FBM trajectories in the presence of localisation errors.

## A. Analytical predictions

Let us start by introducing the mathematical description of the two localisation errors:

(i) The static error is usually modelled as an additive noise term, thus we denote with  $Y_t$  the joint stochastic process of the form

$$Y_t = X_t + e_t, \tag{6}$$

where  $X_t$  is a pure FBM trajectory and  $e_t$  is the static error due to an imperfect measurement. In a standard fashion [61, 62], we stipulate that  $e_t$  is given by the stationary Ornstein-Uhlenbeck (OU) process

$$e_t = \int_{-\infty}^t d\tau e^{-(t-\tau)/\tau_0} \zeta_\tau, \qquad (7)$$

where  $\tau_0$  is the characteristic relaxation time and  $\zeta_t$  is a Gaussian white noise with zero mean and covariance  $\overline{\zeta_t \zeta_{t'}} = 2\sigma_e^2 \delta(t - t')$ . Moreover, it is commonly assumed that  $\tau_0 \ll \Delta t$ , where  $\Delta t$  is the temporal resolution of the trajectory. In other words, we suppose that each time when an instantaneous position  $X_t$  is recorded, the latter is specified up to a random "error" with the distribution

$$P(e_t) = \sqrt{\frac{1}{4\pi\sigma_e^2\tau_0}} \exp\left(-\frac{e_t^2}{4\sigma_e^2\tau_0}\right),\tag{8}$$

and is independent of the previous measurements.

(ii) The dynamic error depends on the acquisition or exposure time  $\tau_e$ , such that the acquired position  $Y_t$  can be written as  $\overline{X_t} = (1/\tau_e) \int_0^{\tau_e} X_{t-\xi} d\xi$ , meaning that we cannot resolve the particle motion below  $\tau_e$ .

Note that the parameters  $\sigma_e$  and  $\tau_0$  for the static error and  $\tau_e$  for the dynamic error are characteristic of the experimental setup and thus they are usually known quantities when analysing SPT experiments.

As treating the dynamic error within the singletrajectory PSD framework is quite tedious and involved, we limit our analytical study to the joint process (6) in which only the static error is present and leave the dynamic error for numerical study, see "Simulations" subsection in Methods.

Selecting the process in (6) and performing the singletrajectory spectral analysis as described above we obtain our central result,

$$\gamma_Y^2(f,T) = \frac{\sigma_Y^2(f,T)}{\mu_Y^2(f,T)} = \frac{\sigma_X^2 + \sigma_{\rm OU}^2 + 2\mu_X \mu_{\rm OU}}{\left(\mu_X + \mu_{\rm OU}\right)^2}, \quad (9)$$

expressing the coefficient of variation of the singletrajectory PSD of the joint process  $Y_t$  via the first moments and the variances of the spectral densities of its constituents. In particular, for the OU process under the assumption stated above, we have  $\mu_{\rm OU} \sim 2\sigma_e^2 \tau_0^2$  and  $\sigma_{\rm OU}^2 \sim 4\sigma_e^4 \tau_0^4$  (see Supplementary Note 1). The results for the FBM process are dependent on H and are in general quite involved. We report here just the asymptotic trends and refer the interested readers to [65] for more details,

$$H < 1/2: \ \mu_X(f,T) \ \sim \frac{2c_H D}{f^{2H+1}},$$
 (10a)

$$H = 1/2: \ \mu_X(f,T) \sim \frac{4D}{f^2},$$
 (10b)

$$H > 1/2: \ \mu_X(f,T) \sim \frac{2D}{f^2} T^{2H-1},$$
 (10c)

and  $\sigma_X^2(f,T) \sim 4D^2 \left(\frac{c_H^4}{f^{4H+2}} + \frac{2c_H}{f^{2H+3}}T^{2H-1} + \frac{2}{f^4}T^{4H-2}\right)$ , where  $c_H = \Gamma(2H+1)\sin(\pi H)$ , and  $\Gamma(z)$  is the Gamma function [65]. Note that in the case of superdiffusion for fixed T the  $1/f^2$  trend could erroneously lead us to the conclusion of having standard diffusion. In addition, the superdiffusive result also shows a dependence on T, a clear feature of ageing, that helps in differentiating it from normal diffusion.

The limiting value at high frequencies of the coefficient of variation obtained in (9) is then given by

$$H < 1/2: \quad \gamma_Y^2(f, T) \sim 1,$$
 (11a)

$$H = 1/2: \quad \gamma_Y^2(f, T) \sim 1 + \frac{1}{4} \left( 1 + \frac{\sigma_e^2 \tau_0^2}{2D} f^2 \right)^{-2}, \quad (11b)$$

$$H > 1/2: \quad \gamma_Y^2(f,T) \sim 1 + \left(1 + \frac{\sigma_e^2 \tau_0^2}{D} \frac{f^2}{T^{2H-1}}\right)^{-2}.(11c)$$

Thus,  $\gamma_Y$  is completely independent of the static noise for subdiffusion, while for normal and superdiffusion correction terms enter. In these, the limit of zero frequency leads us back to the values in absence of noise, that is  $\gamma_Y^2(f = 0) = 2$  (see Supplementary Note 2 for more details). Note that for superdiffusion the limit of long measurement times also leads to the noise-independent value of  $\gamma_Y$ .

#### B. Analysis of simulation data

We start our discussion with results from analytical predictions and simulations (see "Analytical predictions" and "Simulations" subsections in Methods). Results from 1D simulations are shown in Fig. 1. The main goal of this analysis is to elucidate the separate contributions of the two localisation errors, i.e. static and dynamic, in the study of the coefficient of variation  $\gamma$ . Results from simulations in 2D are reported in Fig. 2. The latter are obtained following a procedure that imitates a real experiment (see "Simulations" subsection in Methods for more details) and thus provide more realistic results to be compared with the ones showed below from experiments.

In Fig. 1, panels (a)-(b) we immediately observe that the limiting value of  $\gamma$  in the case of subdiffusive FBM (H = 1/3) is not affected by any of the two measurement noises. In the presence of static error only this result was proved also analytically in Eq. (11a).

For the normal diffusive case (H = 1/2), in Fig. 1, panels (c)-(d), the situation differs distinctly from the subdiffusive case. Namely, the limiting value of  $\gamma$  is affected by both types of measurement noise, resulting in two opposite effects. On the one hand, the static localisation error leads to a drop in the value of  $\gamma$  (Fig. 1, panel (c)). In particular, from the analytical result Eq. (11b) one can see that when the ratio  $\sigma_e^2/D$  grows,  $\gamma$  decreases, approaching the new limiting value  $\gamma = 1$  at very high frequencies. On the other hand, from our numerical study in Fig. 1, panel (d) one can see that the dynamic localisation error causes an increase in the limiting value of  $\gamma$ . The greater  $\tau_e$  the smaller the value of fT at which we observe a deviation from the pure value  $\gamma = \sqrt{5}/2$ .

For superdiffusive FBM, the results are reported in Fig. 1, panels (e)-(f), showing that the limiting value of  $\gamma$  is mostly affected by the presence of static localisation errors. The latter, similarly to the diffusive case, leads to a drop in the limiting value of  $\gamma$ . In particular, if we look at the explicit analytic expression reported in Eq. (11c) we see that upon increasing the ratio  $\sigma_e^2/D$  the coefficient of variation drops, until it reaches the new limiting value  $\gamma = 1$ , as for BM ( $\alpha = 1$ ). Moreover, in this case the trajectory length T also turns out to be a key parameter in determining the final trend of  $\gamma$ : for very long trajectories the presence of static measurement noise becomes less and less influential. For the dynamic error instead we see from Fig. 1, panel (f) that, regardless of the value of  $\tau_e$ , the trend of  $\gamma$  does not present any relevant change.

In general, we observe that localisation errors affect the trend of  $\gamma$  for normal diffusion and superdiffusive FBM only. For both cases ( $\alpha = 1$  and  $\alpha > 1$ ), when the variance  $\sigma_e$  of the static error increases, the drop of  $\gamma$  to the ultimate value 1 occurs at progressively lower values of fT. Instead, when changing the key parameter of the dynamic error ( $\tau_e$ ), the trend of  $\gamma$  is affected only in the case of normal diffusion, showing an increase in its limiting value.

The analysis of numerically obtained FBM data in 2D with different localisation errors is reported in Fig. 2. As can be seen, these results agree with the discussion for the case of the 1D simulations. On the one hand, in the case of subdiffusive FBM the coefficient of variation is insensitive to any of the localisation errors, always converging to the prediction  $\gamma = 1$ . Therefore, this meas-



Figure 1: One-dimensional fractional Brownian motion (FBM) trajectories. We show the coefficient of variation from Monte Carlo simulations of one-dimensional FBM trajectories in the presence of localisation noise. We set the generalised diffusion coefficient to D = 1 and the Hurst exponent to (a)–(b) H = 1/3 (subdiffusion), (c)–(d) H = 1/2(normal diffusion) and (e)–(f) H = 2/3 (superdiffusion). Panels (a), (c) and (e):  $n = 10^4$  realisations consisting of  $N = 2^{23}$  discrete time steps with  $\Delta t = 1$ from the joint process defined in (6), for static error only. The dashed lines represent the expected high-frequency asymptotic trend reported in Eqs. (11a) to (11c) for the different values of  $\sigma_e$  and  $\tau_0 = 1$ . Panels (b), (d) and (f):  $n = 10^4$  realisations consisting of  $N = 2^{14}$  final steps with  $\Delta t = 1$  obtained for pure FBM (black) and in the presence of dynamic error with  $\tau_e$ . The dashed lines represent the limiting value at high-frequencies for pure FBM. Note that  $\gamma$  is reported as a function of fT, thus the limiting values obtained here for high-frequencies are also valid for the case of fixed f and large T.

ure is a very robust mean to explore whether tracking data indeed show a subdiffusive behaviour. On the other hand, diffusive and superdiffusive FBM data do not appear to be very sensitive to the presence of dynamic noise, but their results change dramatically when static noise is present, causing a clear drop in the value of  $\gamma$  at high

frequencies.



Figure 2: Two-dimensional fractional Brownian motion (FBM) trajectories. We show the time-averaged mean squared displacements (TA-MSDs) and the coefficient of variation from Monte Carlo simulations of two-dimensional FBM trajectories in the presence of localisation noise. (a) Representative time-averaged mean squared displacements (TA-MSDs) of individual two-dimensional FBM trajectories created with H = 0.3 (red circles), H = 0.5 (black diamonds), and H = 0.7 (blue squares) follow the anticipated power-law scaling (grey dashed lines) for sufficiently long lag times  $\tau$ . Ensemble averages of TA-MSDs are superimposed as coloured full lines. For short lag times, significant deviations due to a dominant dynamic localisation error ( $n_p = 900$ , filled symbols) or a dominant static localisation error ( $n_p = 50$ , open symbols) are visible. These may significantly perturb the extraction of the TA-MSD's scaling exponent,  $\alpha = 2H$ . (b) The coefficient of variation  $\gamma$ , for the case of a dominant dynamic localisation error (filled symbols) converges towards the predicted values  $\gamma = 1, \sqrt{5}/2$  and  $\sqrt{2}$  (highlighted by the coloured dashed lines). While the subdiffusive case (red) converges rapidly, normal diffusion and superdiffusion (black and blue, respectively) may need longer trajectories to eventually reach the predicted value. In contrast, for a dominant static localisation error (open symbols) only the subdiffusive case (red) is in agreement with the predicted value  $\gamma = 1$  whereas normal diffusion and superdiffusion (black and blue) are very sensitive to this perturbation.

#### C. Analysis of experimental data

Analytical and numerical results are nicely confirmed by the analysis of experimental data. Fig. 3 shows results from subdiffusive tracking data on (i) telomeres in the nucleus of mammalian culture cells [72], and (ii) pgranules in the cytoplasm of *C. elegans embryos* [71], all distinctly displaying a convergence to  $\gamma = 1$ , fully consistent with our predictions.

Experimental data obtained by tracking the motion of beads in water, reported in Fig. 4, show some nice results for the normal-diffusion regime. For the pure experimental data (base-line) we can see an increase in the value of  $\gamma$  at high frequencies. We showed that this deviation from the expected value is due to the effect of the dynamic error. Moreover, if in a post-analysis we artificially increase the static localisation error, we observe that the value of  $\gamma$  starts decreasing upon increase of  $\sigma_e$ , approaching the new limiting value  $\gamma = 1$  (red dashed line in Fig. 4). These observations are fully in line with our predictions.

We now move to the analysis of superdiffusive experimental data. Cytoskeleton fluctuations were measured by attaching RGD-coated beads to the surface of human alveolar epithelial cells (see sketch in Fig. 5, panel (a)). The beads were connected to the actin cytoskeleton and rearranged by internal molecular motors. Given that molecular motor activity depends on temperature, the data show a marked superdiffusive behaviour at high temperatures ( $\geq 29^{\circ}$ C, green triangles and blue circles in Fig. 5,



Figure 3: Coefficient of variation obtained from experimental subdiffusive trajectories with a marked anti-persistence. Results from two datasets are shown: experimental tracking of p-granules in *C. elegans* embryos (open red circles) and telomeres in the nucleus of mammalian culture cells (filled red circles). They both comply with the expectation for sub-diffusive data that  $\gamma$  converges to unity (indicated by the black dashed line). In both cases, the MSDs contained a non-negligible static localisation error.

panel (b)). Interestingly, at low temperatures ( $\leq 21$  °C) the data show that cytoskeleton fluctuations transition from a subdiffusive behaviour at short timescales to a superdiffusive behaviour at longer timescales (black squares in Fig. 5, panel (b)). This crossover can be explained



Figure 4: Coefficient of variation obtained from diffusive data of beads in water. The base-line is marked in blue. Static localisation errors are artificially added to the data to obtain the other two curves. The red and black dashed lines indicate  $\gamma = 1$  and  $\sqrt{5}/2$ , respectively, which are the values to which  $\gamma$  converges in the case of subdiffusion and normal diffusion, when no error is present.

by the Arrhenius dependence of the activity of molecular motors on temperature [73]. Using the coefficient of variation  $\gamma$  to assess the diffusion regime, we observe that cytoskeletal fluctuations obtained at high temperatures  $(29 \text{ and } 37 \circ \text{C})$  display a robust superdiffusive behaviour at all different timescales. The subdiffusive to superdiffusive crossover at 13 and 21  $^{\circ}$  C is well captured as  $\gamma$ approaches 1 for frequencies > 20 rad/s (Fig. 5, panel (c)). At longer timescales the value of  $\gamma$  indicates superdiffusive behaviour, showing that at low temperatures, molecular motors are active at longer timescales. From the analysis of this dataset we can draw two conclusions. First, we see that there is no statistically relevant effect due to localisation errors. Second, we observe that the value of  $\gamma$  is very sensitive to crossovers between different diffusive regimes, confirming that this quantity is useful for the statistical analysis of experimental data.

Finally, we consider the diffusion of nanoparticles in the cytoplasm of human Mesenchymal stem cells. In Fig. 6, panel (a) we report the MSD showing a mildly superdiffusive ( $\alpha = 1.23$ ) regime, in agreement with the fact that the nanoparticles are embedded into the active intracellular environment. However, the MSD trend is strongly affected by the presence of the static localisation error, making the fit less reliable. By calculating the coefficient of variation, reported in Fig. 6, panel (b), we clearly confirm the superdiffusive trend, as  $\gamma$  converges nicely to the analytical value for superdiffusion regime, that is  $\sqrt{2}$ , without displaying large deviations due to the static error.

# III. CONCLUSIONS

Departures from the Brownian behaviour of diffusive processes are observed in a wide variety of systems of practical interest across many disciplines, and these phenomena call for explanations and understanding of the underlying physical processes and microscopic mechanisms. Without such a knowledge one cannot fully comprehend and reach a full picture of the phenomena. Concurrently, a conclusion that the dynamics is indeed anomalous relies on proper data treatment, the size of statistical samples, blurring measurement errors and errors incurred by the fitting procedures, or due to transients obscuring the true scaling exponents. It is therefore indispensable to have at hand robust methods allowing one to reach justified, sound conclusions on the dynamics.

In this work we test the coefficient of variation  $\gamma$  of the single-trajectory power spectral density and show that it represents a valuable method towards a robust criterion for anomalous diffusion. We combine analytical, numerical and experimental studies of diffusive dynamics in very diverse systems, to demonstrate how the values of  $\gamma$  for trajectories belonging to FBM are affected by the presence of localisation errors. Within such a combined effort, numerical simulations which imitate real experiments performed here serve us to elucidate the relative contributions of static and dynamic measurement noise in a controllable way, and therefore to prove our theoretical results and concepts. A further comparison with experimental data obtained for rather diverse systems permits us to verify the predicted trends and thus obtain a fully comprehensive picture.

Apart from the case  $f \to 0$  in which the coefficient of variation  $\gamma(f,T)$  converges to the universal value  $\sqrt{2}$ . we found that our results vary depending on the specific diffusive regime and on the kind of localisation error. In particular, the coefficient of variation represents a very robust way to define whether tracking data show a subdiffusive behaviour. This can be of decisive help in data analysis, in particular, when the deviation of the anomalous exponent from unity is small and thus the fitting of the MSD can produce misleading results. Conversely, diffusive and superdiffusive data are more sensitive to measurement noise. In these regimes the value of  $\gamma$  displays a clear drop at high frequencies in the presence of a static localisation error, which is corroborated by both numerical and experimental data, while the effect of a dynamic error appears to be relevant for the normal-diffusive regime only, causing an increase in the limiting value of  $\gamma$ at high frequencies. As well, this theoretical prediction is confirmed by our numerical and experimental results. Thus, in the case of superdiffusion the criterion remains very robust in the presence of dynamic error while in general it is not when static localisation error is present. Nevertheless, the analytical expression obtained for the correction term still allows us to control the effect of the measurement error in this case. Finally, the criterion appears to be the least robust for normal diffusion, which is



Figure 5: Cytoskeleton fluctuations of living cells exhibit superdiffusive behaviour. (a) Sketch of the experimental system for microbeads attached to the surface of epithelial cells (see the "Experiments" subsection in the Methods for more details) together with a representative experimental trajectory. (b) MSD at varying temperatures. The straight lines show that the dynamics are superdiffusive with 2*H* between 1.5 and 1.7, depending on temperature. (c) Coefficient of variation of the power spectrum of the motion of the surface-bound microbeads. The dashed lines indicate  $\gamma = 1$ ,  $\sqrt{5}/2$ , and  $\sqrt{2}$ , the predicted limiting values of  $\gamma$  for the different diffusion regimes.



Figure 6: Mean squared displacement (MSD) and coefficient of variation for superdiffusive nanoparticles in hMSCs. (a) MSD showing a superdiffusive regime with large deviations due to the presence of the static localisation error. (b) The coefficient of variation confirms the superdiffusive trend.

affected by both static and dynamic localisation errors. Arguably, the observed trends in  $\gamma$  can be used also to post-process the data by adding static and dynamic error to explore the limiting values of  $\gamma$  as function of noise intensity.

The application of our method to diverse experimental systems presented in our paper evidences that it provides a very robust tool for the analysis of the anomalous character of random motion. In conjunction with the MSD analysis, it will permit to make conclusive statements on the actual departures from standard diffusive motion, even in the presence of unavoidable errors in experimental measurements, and thus to point towards the necessity to understand the actual underlying physical mechanisms. Apart from a stand-alone criterion our power spectral method will also represent an important ingredient in decision trees and feature-based neural network approaches.

#### IV. METHODS

#### A. Simulation methods

We perform simulations of FBM trajectories in 1D and 2D in order to complement and support our analytical predictions as well as the analysis of experimental data. The simulations are performed in Python for the 1D case and in Matlab for the 2D case.

## 1. 1D case

FBM trajectories of length  $T = N \times \Delta t$ , where N corresponds to the total number of time steps and  $\Delta t$  to the discretisation time step, are generated for different values of the Hurst index H to explore all 3 regimes via the *fbm* package in Python. In order to include the dynamic error, given a fixed exposure time  $\tau_e$  corresponding to  $n_e = \tau_e/\Delta t$  time steps, we first simulate longer FBM trajectories with a total number  $N_e = N \times n_e$  of time steps. Then, from each of these trajectories a new trajectory is obtained, whose points are given by the av-

erage over  $n_e$  subpoints of the original trajectory, i.e.,  $\overline{X}(t_i) = \frac{1}{n_e} \sum_{j=0}^{n_e-1} X(t_i - j\Delta t)$ . As far as the static error is concerned we use its definition (7) and generate stationary OU trajectories with relaxation time  $\tau_0 = \Delta t$ and varying noise amplitude  $\sigma_e$ .

#### 2. 2D case

An ensemble of M = 100 two-dimensional FBM trajectories, each with N = 2500 positions covering a total time  $T = N \times \Delta t$  with  $\Delta t = 0.1$  s was created with the Matlab routine wfbm for the Hurst coefficients H = 0.3. 0.5, and 0.7, hence exploring FBM from the subdiffusive to the superdiffusive regimes. Mean step sizes within  $\Delta t$ along each coordinate were chosen to be 0.01  $\mu \mathrm{m}.$  To account for the dynamic and static localisation errors, each time step was subdivided into  $n_e = 10$  substeps during each of which  $n_p$  positions according to a diffractionlimited Gaussian PSF, centred at the particle position at that time, were calculated. To this end, initial FBM tracks had a length  $i = 1, \ldots, Nn_e$  and at each substep location  $x_i$ , a total of  $j = 1, \ldots, n_p$  positions  $x_{i,j} = x_i + \xi_j$ were produced, where  $\xi_i$  are Gaussian random numbers with zero mean and standard deviation  $\sigma = 0.220 \ \mu m$ . From this, track positions were determined via

$$X_k = X(k\Delta t) = \frac{1}{n_e n_p} \sum_{i=(k-1)n_e+1}^{kn_e} \sum_{j=1}^{n_p} x_{i,j}.$$
 (12)

For  $n_p < 100$ , this procedure creates a dominant static localisation error whereas for  $n_p \gg 100$  only a dynamic localisation error is seen.

#### B. Experimental systems

To assess the applicability to experimental data of the coefficient of variation analysis in the presence of localisation errors, we analysed experimental data displaying different diffusive regimes. In what follows we report a short description of the experimental systems.

## 1. Subdiffusive data

Experimental tracking data for p-granules in C. elegans embryos. The data were obtained and analysed as described in [71]. As shown before, p-granule trajectories have a noticeable static localisation error that perturbs, e.g., the velocity autocorrelation function. Here, only trajectories with N = 100 positions at a time increment  $\Delta t = 210$  ms with a subdiffusive TA-MSD scaling were considered. Trajectories with scaling exponents  $\alpha \in [0.7, 0.9], \alpha \in [0.5, 0.7],$  and  $\alpha \in [0.3, 0.5]$  were grouped into three distinct sets. For each,  $\gamma$  was calculated as a function of fT, and the average of these was used for Fig. 3 to soften fluctuations induced by the fairly short trajectories.

Trajectories for telomeres in the nucleus of mammalian culture cells. The data were obtained similar to our previous work [72]: U2OS cells (DSMZ Cat# ACC-785, RRID:CVCL 0042) were cultured as described and telomeres were highlighted by transient transfection with a plasmid for GFP-tagged TRF-2 (24 h prior to microscopy, using Lipofectamine3000 according to the manufacturer's protocol). For live-cell microscopy, cells were plated 24 h prior to transfection in 4-well  $\mu$ -slide microscopy chambers; 15 min prior to imaging, the medium was changed to MEM without phenol red supplemented with 5% FCS and 5% HEPES. Imaging was performed with a customised spinning-disk confocal microscope based on a DMI 4000 stand (Leica Microsystems, Germany) with a custom-made incubation chamber, a CSU-X1 spinning-disk unit (Yokogawa Microsystems, Japan), an HC PL APO 100x/1.40NA oil immersion objective, and an Evolve 512 EMCCD camera (Photometrics, USA). Samples were illuminated at 488 nm and fluorescence was detected in the range 500-550 nm. Trajectories were recorded at a time increment  $\Delta t = 110 \text{ ms}$ and only trajectories with at least N = 1000 positions were retained (and trimmed to the same length if trajectories were longer). In line with our previous finding, trajectories featured an anti-persistent subdiffusion with an average scaling exponent of  $\alpha \approx 0.55$ .

In both cases, the generalised diffusion coefficients of individual trajectories varied log-normally. To soften the influence of this locus- and particle-dependent prefactor in the PSD analysis, each trajectory was normalised in each coordinate by its respective root-mean-squared step taken within  $\Delta t$  before calculating  $\gamma$ .

## 2. Diffusive data

We analysed the motion of 1.2  $\mu$ m-sized polystyrene beads in aqueous solution as representative experimental data for Brownian motion. Namely, we suspended the beads in phosphate-buffered saline with 1% bovine serum albumin and 0.05% Tween 20 to avoid aggregation, and introduced the suspension into a flow cell chamber. Subsequently, the flow cell chamber was sealed for imaging. The beads were imaged at 100 frames per second in an inverted microscope with a 40x objective (Olympus PlanApo, N.A. 0.95) and a sCMOS camera (Andor Zyla 4.2). Bead tracking in the plane was performed in Lab-View using a cross-correlation based tracking algorithm. A set of 150 trajectories, each consisting of 4096 frames, was used in the analysis.

#### 3. Superdiffusive data

Cytoskeleton fluctuations of living cells at different temperatures [73]. Cytoskeleton fluctuations were measured by tracking the trajectory of refractive microbeads attached to the surface of human alveolar epithelial cells (A549). The microbeads were previously coated with Arginine-Glycine-Aspartic acid (RGD) containing peptide (Peptide 2000, Integra life Sciences, San Diego, CA) to link the probe to the actin cytoskeleton through integrin membrane receptors. The movement of these beads was sensitive to a wide range of cytoskeleton manipulations including actin polymerisation/depolymerisation drugs, actomyosin relaxation, cell stretching, temperature changes, and ATP depletion [74] indicating that spatial fluctuations of microbeads linked to integrin membrane receptors reflect intrinsic cytoskeleton dynamics.

Microbead positions were tracked at 40x magnification using an inverted microscope (TE-2000E, Nikon, Japan) equipped with a charge coupled device (CCD) camera (Orca, Hamamatsu, Japan). The spontaneous microbead movement was tracked for 200-400s at a sampling rate of 5 Hz. The position of the microbead was determined with subpixel resolution by computing the microbead centroid with an intensity-weighted average algorithm implemented with a custom-made software (LABVIEW, National Instruments, U.S.). Data were corrected for the drift of the stage of the microscope, which was computed as the average change in the position of all microbeads within the field of view.

The temperature dependence of cytoskeleton fluctuations was measured by heating or cooling the microscope stage with a microincubator system (HCMIS MicroIncubator System, ALA Science, Westbury, NY) and closed-loop control. The sample temperature was measured with a negative temperature coefficient thermistor (332 Temperature Controller, Lakeshore, Westerville, OH). Measurements were taken in n = 6 wells (~ 20 microbead/well) per temperature.

Human alveolar epithelial cells (A549) (cell line CCL-185 ATCC, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 1 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 2  $\mu$ g/ml amphotericin B (all from GIBCO, Gaithersburg, MD), 10% inactivated fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), and buffered with HEPES (Sigma, St. Louis, MO). One day before experiments cells were harvested with a brief exposure to trypsin-EDTA (Sigma) and plated (900 cells/mm<sup>2</sup>) on collagen-coated wells.

Diffusion of nanoparticles in the cytoplasm of human Mesenchymal stem cells. Here we tracked yellowgreen fluorospheres of size 100 nm injected carefully in the cytoplasm of human mesenchymal stem cells (hM-SCs). The fluorospheres (FluoSpheres<sup>TM</sup> ThermoFisher, Cat. No: F8803) were negatively charged (carboxylatedmodified) polystyrene beads that are suitable for intracellular tracking. Samples were prepared by diluting the suspension to 2 mg/ml concentration after 20 minutes sonication of the stock solution to ensure even dispersion of the particles in the solution. The diluted solution subsequently was vortexed for two minutes for optimised mixing and then loaded via a microloader pipette (Eppendorf) in manufactured glass capillaries appropriate for microinjection (Femtotips<sup>®</sup> Eppendorf Cat. Nr.: 5242952008). Their microinjection was executed at room temperature using a micromanipulation system (Eppendorf) at controlled pressure. Immediately after the injection, imaging was performed 100 s at room temperature with an Olympus IX81 inverted microscope using a Olympus UPLSAPO40X/0.95 Objective and a Hamamatsu Orca-2 camera. At each frame of the resulting video files the particles were identified and their movement were tracked using a publicly available Python package which also provides tools to spot the candidate features based on high intensity matches, filtering and different type of corrections such as drift correction [75].

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