From sub- to superdiffusion: fractional Brownian motion of membraneless organelles in early C. elegans embryos

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Abstract
Fractional Brownian motion (FBM) is a prevalent Gaussian stochastic process that has frequently been linked to subdiffusive motion in complex fluids, e.g. inside living cells. In contrast, examples for a superdiffusive FBM in complex fluids are sparse, and a covering of all FBM regimes in the same sample is basically lacking. Here we show that membraneless organelles in the single-cell state of C. elegans embryos, so-called p-granules, constitute an experimental example in which the whole range of FBM processes, from the sub- to the superdiffusive regime, can be observed. The majority of p-granules is subdiffusive, featuring an antipersistent velocity autocorrelation function (VACF). A smaller fraction of trajectories shows normal diffusion or even superdiffusion with a persistent VACF. For all trajectories, from sub- to superdiffusive, the VACF, its characteristic values, and the trajectories’ power-spectral density are well matched by FBM predictions. Moreover, static localization errors, a frequent problem in single-particle tracking experiments, are shown to not affect the conclusion that p-granule motion is best described by FBM from the sub- to the superdiffusive regime.

1. Introduction
Elucidating and quantifying transport phenomena in small, fluctuation-dominated systems is a topical problem in the wider area of soft-condensed matter and non-equilibrium physics. This holds true in particular when aiming to understand self-organization processes in living matter, e.g. in the context of intracellular segregation processes. A prominent example for the latter is observed prior to the first cell division in the embryo of the nematode C. elegans, where spatial protein gradients emerge during the single-cell stage [1–7], eventually defining the anterior-posterior (AP) body axis by establishing biochemically distinct poles in the ellipsoidally shaped cell [8]. During the same developmental stage, membraneless organelles, called p-granules, become enriched in the posterior part of the cell, designating the emerging posterior daughter cell to become the precursor of the developing animal’s germline [8].

P-granules have been shown to be droplet-like assemblies of proteins and nucleic acids, whose formation and posterior enrichment rely on a (de-)condensation process that is guided by the aforementioned protein gradients along the AP-axis [9, 10]. Due to their composition and the lack of an engulfing membrane, p-granules are prototypical examples for the wide class of membraneless organelles in eukaryotic cells [11]. Although p-granules were not seen to migrate over long distances from the anterior to the posterior cell pole [9], they still show a vivid movement in the cell. Due to a multitude of active processes in the cell, from short-range motor-driven transport along the cytoskeleton up to a transient cytoplasmic streaming on larger length and time scales [12], p-granules can be expected to not just move by thermal diffusion. So far, however, it has remained unclear how much of the motion of individual p-granules is a consequence of simple diffusion or actively driven processes.

Establishing ties between the special cases of purely ballistic transport, thermally driven diffusion, and complete immobilization, fractional Brownian motion (FBM) is a prevalent Gaussian stochastic process.
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R Benelli and M Weiss

[13] that is characterized by the so-called Hurst coefficient $H$. For $H < 1/2$, an anti-correlated memory kernel leads to an antipersistent diffusion process, whereas for $1/2 < H < 1$ a persistent random walk is obtained. As a physical process, FBM may be obtained from a fractional Langevin equation that includes temporally correlated noise [14]. At $H = 1/2$, the memory kernel becomes Markovian and normal Brownian diffusion is regained. In all cases, the mean square displacement (MSD) of trajectories grows with time as a power law, i.e. $\langle r(t)^2 \rangle \sim t^\alpha$ with $\alpha = 2H$, yielding a sub- or superdiffusive random walk for $\alpha < 1$ or $\alpha > 1$, respectively. In fact, diffusional motion in complex fluids frequently displays subdiffusive characteristics (see [15, 16] for an overview), often related to a viscoelastic behavior of the fluid with trajectories having all features of an FBM process with $H < 1/2$. FBM-like subdiffusion of tracer particles has been reported, for example, for the cytoplasm [17–19] and nucleoplasm [20] of living cells, and also in artificial crowded fluids [21, 22]. In fact, crowding on many different length scales, i.e. from macromolecules up to vast endomembrane systems, appears to equip cellular fluids with a viscoelastic character [23], inducing an antipersistent memory kernel for diffusional motion (see also [15, 16] for more information on FBM and experimental observations). In comparison, experimental examples and evidence for a superdiffusive FBM in complex fluids, i.e. persistent successive steps that are not simply a ballistic motion, are considerably more sparse [24], and a covering of all FBM regimes in the same sample is basically lacking.

Here we show that p-granules in the single-cell state of C. elegans embryos constitute an experimental example in which the whole range of FBM processes, from the sub- to the superdiffusive regime, can be observed. In particular, using single-particle tracking (SPT), we find that the vast majority of p-granules shows a subdiffusive scaling of the MSD that is associated with an antipersistent velocity autocorrelation function (VACF). A smaller fraction of trajectories features a normal or even clearly superdiffusive MSD with a persistent VACF. For all trajectories, from sub- to superdiffusive, the characteristic VACF values are well captured by an FBM prediction that is solely based on the extracted MSD scaling exponents. Also FBM predictions for the trajectories’ power-spectral density are well matched. Quantifying and correcting for the influence of static localization errors, a frequent problem in SPT experiments, is shown to not affect the conclusion that p-granule motion is best described by FBM from the sub- to the superdiffusive regime.

2. Materials and methods

2.1. Sample preparation and microscopy

In this study we have used C. elegans strain JH2842 (pgl-1::GFP) [25], provided by the caenorhabditis genetics center. In this strain, the p-granule constituent protein PGL-1 is tagged with a green fluorescent protein (GFP). Worms were cultivated on nematode growth medium plates at 24°C for proper expression; measurements were performed at 20.5°C. Sample preparation was done as described before [7, 26–30] by dissecting young adult worms to access freshly fertilized embryos. Embryos were placed in a 10 $\mu$l drop of M9 buffer on coverslips ($24 \times 60$ mm #1, Menzel-Glaser, Germany) and 2.5 $\mu$l of a solution of 20 $\mu$m beads (Polybead Microspheres, Polysciences Inc., USA) were added to prevent smashing of the embryo when placing a second coverslip ($20 \times 20$ mm #1, Menzel-Glaser, Germany) on top. Borders were sealed with 90°C prewarmed vaseline (Unilever, UK). Imaging was performed with a customized spinning-disk setup consisting of a Leica DMI 6000 microscope body (Leica Microsystems, Germany) equipped with a CSU-X1 (Yokogawa Microsystems, Japan) spinning disk unit. Single-plane images were taken with a Hamamatsu Orca Flash 4V2.0 camera (Hamamatsu, Japan) and an HCXPL APO 100x/1.4NA oil immersion objective. For sufficient imaging quality, the frame time was adjusted between different embryos, resulting in frame times of $\Delta t = 110$ ms, $\Delta t = 160$ ms, or $\Delta t = 210$ ms. GFP-tagged proteins were excited at 488 nm and the fluorescence was detected in the range 500–550 nm.

2.2. SPT and trajectory analysis

Trajectories were extracted from images with FIJI/TrackMate [31]. To facilitate a proper detection of p-granules, images were first processed with ilastik, an interactive machine-learning tool for image analysis [32]. To this end, the data was exported from FIJI to ilastik and the interactive pixel classification was used to segment the granules from the cytoplasmic background noise. Briefly, all pixel features with $\sigma < 3.5$ were used, yielding a total of 25 features. The machine learning algorithm was trained with three different images at early, intermediate and late times to compensate bleaching effects. Here, for each picture about ten hand-drawn annotations for the granules and the background were sufficient. The pixel prediction probabilities were exported and binarized with FIJI’s automated minimum-thresholding. As a further input for TrackMate, the radius of the granules was estimated via visual inspection to be around 1.5 $\mu$m. Tracking was performed using the Laplacian-of-Gaussian algorithm (blob diameter set to 1.5 $\mu$m, threshold set to a nonzero value and sub-pixel localization switched on). No additional filters were applied to the detected
Figure 1. (a) Representative image of a *C. elegans* embryo (two-pixel median-filtered) just before the pronuclear meeting with PGL-1 protein fluorescence highlighted via the lookup table ‘mpl-inferno’; bright yellow spots highlight p-granules, cytoplasmic proteins contribute a dim red/blue background. Parental pronuclei are visible as dark round spots. Letters A and P highlight the anterior and posterior cell pole, respectively, indicating the embryo’s AP-axis. Close-ups in the neighboring column show three typical p-granule trajectories (red), superimposed to the fluorescence image of the trajectory’s first time point (in greyscale for better visibility). (b) Representative examples of TA-MSDs, $\langle r^2(\tau) \rangle_t$, highlight considerable variations between individual p-granule trajectories (color-coded for better visibility). Dashed lines indicate power-laws $\sim \tau$ and $\sim \sqrt{\tau}$ as a guide to the eye.

...spots. Identified particle positions were linked using the linear assignment problem tracker adopted from reference [33]. A maximum linking distance of 1.5 μm was used and no gaps were allowed in the position time series. Only tracks with $N > 70$ positions were retained for further analysis. Beforehand, all tracks were rotated in such a way that the $x$-axis coincided with the AP-axis of the respective embryo. To this end, the main axes of the embryo in lab coordinates were obtained in Matlab via thresholding after binarization, and the angle of rotation was extracted. Subsequent statistical analyses of trajectories were performed in Matlab with custom-written codes that have been checked for proper function via simulation data. A total of 1198 trajectories, acquired in $n = 11$ embryos, comprised at least 50 positions. From these, only those 837 trajectories were retained for further analyses that had $N > 70$ positions and were not rated to represent immobile particles (criterion: time-averaged mean square displacement (TA-MSD) scaling exponent $\alpha > 0.09$). The vast majority of trajectories (591) contained at least $N = 100$ positions and only few (65) featured lengths $N \geq 500$. No distinct trajectory pools (e.g. relations like 'longer trajectories display more often a subdiffusive motion') were discernible. The minimum and maximum real-time lengths of trajectories were 7.81 s and 294.42 s, respectively.

3. Results and discussion

To explore the motion of p-granules, we followed previous approaches in which these prototypical membraneless organelles had been monitored in the one-cell stage of *C. elegans* embryos [9]. In particular, we imaged p-granules via fluorescently tagged constituents, so-called PGL-1 proteins fused to a GFP [25], and determined individual trajectories $r(t) = r_1, \ldots, r_N$ from the acquired images with frame time $\Delta t$ (see figure 1(a) for a representative image and materials and methods for technical details). We have concentrated on the developmental stage near to the so-called pronuclear meeting at which parental pronuclei fuse to form the single-cell state of the embryo. At this stage, cytoplasmic streaming with typical velocities in the range of few microns per minute might be present [12], as discussed below.

As a first measure, we calculated for each trajectory the TA-MSD as a function of the lag time $\tau = k\Delta t$,

$$\langle r^2(\tau) \rangle_t = \frac{1}{N - k} \sum_{i=1}^{N-k} (r_{i+k} - r_i)^2.$$  

(1)

TA-MSDs of p-granules showed considerable variations between individual trajectories, even for lag times $\tau < 1$ s where statistics for the averaging was fairly high (see examples in figure 1(b)). To obtain a quantitative and objective measure for this variation, we performed a linear regression of $\log(\langle r^2(\tau) \rangle_t)$ versus $\log(\tau)$ for each TA-MSD in the interval $0.2 \leq \tau \leq 2$ s, representing a simple power-law fit of the form

$$\langle r^2(\tau) \rangle_t = dK\tau^\alpha.$$  

(2)

Here, $d = 1, 2$ for one- and two-dimensional trajectories, respectively. The prefactor $K$ represents a generalized transport coefficient for each trajectory (with units of an area per fractional time), whereas the scaling exponent $\alpha$ indicates the type of motion, e.g. $\alpha < 1$ for subdiffusion. Using this simple fit approach
neglects any static or dynamic localization error in the position time series that may perturb the MSD scaling for small lag times \[34\]. The associated misinterpretations will be discussed quantitatively below.

As a result of the fit process, we observed a very broad probability density function (PDF) of scaling exponents, \(p(\alpha)\), as shown in figure 2(a). The width of this PDF is considerably larger than the expected statistical variation for short trajectories that emerge from the same random-walk process (see reference \[35\]). In particular, about 65% of all trajectories were clearly subdiffusive (\(\alpha < 0.9\)), about 21% showed mostly normal diffusion (0.9 \(\leq \alpha \leq 1.1\)), and about 14% were superdiffusive (\(\alpha > 1.1\)). The predominant occurrence of subdiffusive p-granules is in line with previous observations on micron-sized objects in the cytoplasm (see, for example, references \[17, 19\]), i.e. it may be seen as the basic mode of motion in the viscoelastic cytoplasm to which any active process may be superimposed. Notably, no significant changes of \(p(\alpha)\) were observed when analyzing the trajectories’ individual coordinates, even though the \(x\)-coordinate was always chosen to point along the AP-axis of the embryo (cf materials and methods). This finding indicates that the AP-axis is not a preferential axis for p-granule motion.

The PDF of generalized transport coefficients, \(p(K)\), obtained from fitting TA-MSDs, also did not vary markedly between individual coordinates and the two-dimensional trajectory (figure 2(b)), featuring an almost lognormal shape with a distinct peak at \(K \approx 0.016 \text{ \mu m}^2 \text{ s}^{-1}\). A slight correlation between \(\alpha\) and \(K\) (black symbols; red dashed line) as obtained from fitting TA-MSDs, also did not vary between different trajectories, the units of \(K\) depend on \(\alpha\). Values of \(K\) should therefore be interpreted as typical area that is explored within 1 s \((K \times 1^3)\).

Figure 2. (a) PDF of scaling exponents, \(p(\alpha)\), obtained from fitting TA-MSDs of individual p-granule trajectories with equation (2) (black histogram). While the majority of trajectories (about 65%) is clearly subdiffusive (\(\alpha < 0.9\)), also a smaller fraction of almost normal and even superdiffusive (\(\alpha > 1.1\)) trajectories are included in \(p(\alpha)\). Analyzing only the trajectories’ \(x\)- and \(y\)-coordinates did not yield significant differences (red and blue symbols, respectively) although the \(x\)-axis always pointed along the AP-axis of the cell. (b) The associated PDF of generalized transport coefficients, \(p(K)\), as obtained from fitting TA-MSDs, features an almost lognormal shape with a pronounced peak around \(K \approx 0.016 \text{ \mu m}^2 \text{ s}^{-1}\) for the two-dimensional trajectories and for their individual coordinates (color code as before). Please note: since anomaly exponents vary between different trajectories, the units of \(K\) depend on \(\alpha\). Values of \(K\) should therefore be interpreted as typical area that is explored within 1 s \((K \times 1^3)\). (c) A slight correlation between \(\alpha\) and \(K\) (black symbols; red dashed line) is consistent with the notion that particles which move more actively will feature a more persistent motion (\(\alpha\) higher) at an elevated transport coefficient (\(K\) higher). (d) Extracting \(\alpha\) and \(c_1(\xi = 1)\) at \(n = 3\) (cf equation (3)) for each trajectory (black symbols) shows an overall good agreement with the FBM prediction (equation (6), red dashed line). This result supports the notion that p-granules motion can be described as an FBM process from the sub- to the superdiffusive regime.

\[ c_1(\tau) = \frac{\langle v(t)v(t+\tau)\rangle_\tau}{\langle v(t)^2\rangle_\tau}, \]
from which the considerably smoother ensemble- and time-averaged VACF

\[ C(\tau) = \langle c_i(\tau) \rangle_E \]  \hspace{1cm} (4)

can be obtained. Here, \( v(t) = [r(t + \delta t) - r(t)]/\delta t \) is the instantaneous velocity, where \( r(t + \delta t) - r(t) \) denotes the spatial increment taken within integer multiples of the frame time, \( \delta t = n\Delta t \). Rescaling the lag time \( \tau = k\Delta t \) with \( \delta t \), i.e. using a dimensionless time \( \xi = \tau/\delta t = k/n \) allows for a direct comparison of experimentally determined VACFs to an analytical prediction for persistent and antipersistent FBM processes [13, 14],

\[ C_{\text{FBM}}(\xi) = \left\{ (\xi + 1)^{\alpha} + |\xi - 1|^{\alpha} - 2\xi^\alpha \right\}/2. \]  \hspace{1cm} (5)

Since FBM processes are ergodic, this expression also holds for the time-averaged VACF of (sufficiently long) individual trajectories, \( c_i(\xi) \). The self-similarity of FBM processes is encoded in equation (5) by the fact that the same VACF value is obtained when varying \( k \) and \( n \) but keeping \( \xi \) fixed. For practical purposes, however, the time scale \( \delta t \) will have to be chosen in such a way that a time-averaging over the trajectory is not spoiled by insufficient statistics (requesting the parameter \( n \) to be small) while still allowing for a fine sampling of \( \xi \) (asking for \( n \) to be large). Balancing these opposing demands, we will mostly concentrate on \( n = 3 \) when analyzing our experimental data.

A direct consequence of equation (5) is the prediction of the characteristic VACF value at \( \xi = 1 \) for all anomaly exponents \( 0 < \alpha < 2 \), that is,

\[ C_{\text{FBM}}(\xi = 1) = 2^{\alpha-1} - 1. \]  \hspace{1cm} (6)

Probing this relation with our experimental data, i.e. extracting \( \alpha \) and \( c_i(\xi = 1) \) at \( n = 3 \) for each trajectory, yields overall a very good agreement with the FBM-derived prediction (figure 2(d)). This result strengthens the evidence that p-granule motion is well described by an FBM process from the sub- to the superdiffusive regime. The good agreement of the experimental data with equation (6) is even more striking when considering that individual time-averaged VACFs for the fairly short trajectories suffer from strong statistical fluctuations due to poor averaging.

Given that FBM is a Gaussian process, we next tested the shape of PDFs for step increments of p-granules taken in a period \( \delta t = n\Delta t \). Deviations from a Gaussian PDF, highlighting a heterogeneous diffusion characteristics, have been reported earlier for several systems [17, 19, 36, 37] and were rationalized by random walks with spatiotemporally fluctuating transport coefficients [38, 39] and/or by systems with spatial disorder [40, 41]. Following this earlier work, we calculated for each trajectory the sequence of step increments \( (\delta x_i, \delta y_i) = (x_{i+n} - x_i, y_{i+n} - y_i) \) with \( i = 1, \ldots, N - n \), and normalized these by their respective root-mean-square values. Since the resulting set of normalized steps did not exhibit significant differences between the x- and y-coordinates, we combined both into a single set of normalized increments, \( \chi \). By construction, the ensemble set of all normalized increments \( \chi \) obtained in this way will follow a standard Gaussian for any choice of \( n \) if the trajectory is due to an FBM process.

Accounting for the different modes of motion, we have pooled the normalized increments of all trajectories according to their anomaly exponents, yielding a set for subdiffusive \((\alpha < 0.9)\), normally diffusive \((0.9 \leq \alpha \leq 1.1)\), or superdiffusive \((\alpha > 1.1)\) trajectories. Since the associated PDFs \( p(\chi) \) were symmetric, we only show \( p(|\chi|) \), also known as van-Hove function, in figure 3. As a result, we observed that all three sub-ensembles feature marked deviations from the anticipated standard Gaussian for steps taken between consecutive frames \((n = 1)\). A simple superposition of two Gaussians with relative weights \( f_1 = 0.85 \) and \( f_2 = 1 - f_1 = 0.15 \) and variances \( \sigma_1^2 = 0.81 \) and \( \sigma_2^2 = (1 - f_1)\sigma_1^2/(1 - f_1) \approx 2.1 \) was sufficient to capture the form of \( p(|\chi|) \) for all modes of motion (see figure 3). Even when lumping all increments into one set, irrespective of the value of \( \alpha \), the same heterogeneous shape in \( p(\chi) \) is seen, hence indicating that the same two processes are driving successive steps in all p-granule trajectories, from sub- to super-diffusive trajectories. In contrast to this finding, step increments between more distant frames \((n = 10)\) showed little to no deviation from the standard Gaussian (figure 3, insets), suggesting that the diffusion heterogeneity subsides and trajectories are driven by a single Gaussian process on longer time scales.

Both observations, i.e. a short-term diffusion heterogeneity followed by a normal Gaussian process on longer time scales, are similar to a previous report on the intermittent motion of other particles in the cytoplasm [19]. Yet, in contrast to this earlier report, we did not observe a significantly nonzero autocorrelation of squared increments that is known to show a characteristic exponential decay for an intermittent switching between two different modes of motion [19, 42]. Therefore, individual p-granules appear to remain in one mode of motion during the observation time of our experiments. While we cannot rule out that an intermittent switching might be observed on time scales shorter than our frame time, it appears more likely that the short-term diffusion heterogeneity of p-granules emerges from a superposition of two independent processes. Given the good agreement of the simple diffusion estimate with the peak in
and with marked deviations from equation (5) at trajectories display positive values for figure 3, is simply due to a pure FBM process to which a smaller fraction of Markovian range of few microns per minute [12] would be too slow a process to explain such kicks. Experimental observation (figure 3, insets). Notably, cytoplasmic streaming with typical velocities in the important, resulting in a convergence to the expected standard Gaussian, in agreement with the superimposed to the otherwise diffusive motion. For longer time scales, these kicks become less and less stochastic motor-driven properties [43], i.e. motor-dependent local force fluctuations were seen to along microtubules, may occasionally bump into p-granules, providing stochastic kicks that are intra-nuclear motion [20]. Thus, nearby ballistic transport events, e.g. molecular motors dragging cargo substantially enhance intracellular motion. Similarly, active random forces were reported to drive sub-ensemble. The resulting VACF curves follow, by and large, the FBM prediction equation (5) for all α ∈ [α0 − 0.1, α0 + 0.1] with varying α0, and averaged their time-averaged VACFs only over the respective sub-ensemble. The resulting VACF curves follow, by and large, the FBM prediction equation (5) for all values of α0; subdiffusive trajectories show a pronounced minimum at ξ = 1 whereas superdiffusive trajectories display positive values for ξ ≥ 1 with an asymptotic decay to zero (see figure 4). However, marked deviations from equation (5) at ξ = 1, most pronounced for α0 ≥ 1, indicate that the underlying trajectories are perturbed by static localization errors. These arise, for example, when retrieving particle positions with a limited number of photons in the image [34], hence decorating each position in the trajectory with additive Gaussian random numbers in every coordinate. These additive perturbations are Markovian and therefore perturb the memory and self-similarity of FBM trajectories, pushing C(ξ = 1) toward zero.

To explore the impact of these perturbations in more detail, we have used an analytical expression for the VACF of FBM random walks that explicitly considers static and dynamic localization errors [34]. The analytical form of this (already normalized) VACF for δt = nΔt and lag time τ = kΔt (eventually defining again a dimensionless time ξ = k/n) reads

\[ C(n, \alpha, k) = \frac{A(n + k, \alpha) - 2A(k, \alpha) + Q}{2A(n, \alpha) - 4\theta(\alpha + 1)(\alpha + 2)} \]  \hspace{1cm} (7)

with

\[ Q = \begin{cases} 2 - \theta(\alpha + 1)(\alpha + 2) & \text{for } k = n \\ A(k - n, \alpha) & \text{for } k \neq n \end{cases} \]  \hspace{1cm} (8)

and

\[ A(x, \alpha) = (x + 1)^{\alpha+2} + (x - 1)^{\alpha+2} - 2x^{\alpha+2}. \]  \hspace{1cm} (9)

While the contribution of a dynamic localization error is included in the VACF via sums of the functions A(x, \alpha), the constant θ in equations (7) and (8) summarizes the influence of the static localization error. For a known FBM process with transport coefficient K and anomaly exponent α this constant is given by...
Let us discuss now, to which extent the perturbations by a static localization offset affect the conclusion that p-granule motion represents an FBM process. Given that all increment PDFs showed a very similar peak in $\Delta \alpha = 0.2$ yields a very good fit to all data, e.g. for $n = 3$ (black lines connecting the discrete values obtained by equation (7)). This finding clearly indicates that our p-granule trajectories are perturbed by a significant static localization offset, requiring also an update of the anomaly exponents found by simple power-law fitting of the MSD (see also main text for discussion).

\[
\theta = \sigma^2/(K\Delta t^\alpha). \quad \text{Here, } \sigma^2 = \text{the variance of the microscope's point-spread function used for image acquisition, divided by the mean number of photons acquired for the tracked object [34]; therefore, } \theta, \sigma^2 \rightarrow 0 \text{ for very good photon statistics. For experimental data, } \theta \text{ rather may be used as an open fit parameter. It is also worth noting that equation (7) is, unlike equation (5), only properly defined at discrete values of } n \text{ and } \xi \text{ since the strict self-similarity of trajectories is perturbed.}
\]

As can be seen in figure 4, equation (7) indeed provides a very good fit to our experimental VACF data for all values of $\alpha_0$ when choosing $\theta = 1.8$ and using updated anomaly exponents $\alpha_0 + \Delta \alpha$ with $\Delta \alpha = 0.2$. Notably, $\theta = 1.8$ yields $\sigma^2 \approx 2K\Delta t^\alpha$, indicating a static localization error in the range of 80 nm when employing again the simple diffusion estimate ($\alpha = 1, K = 0.016 \mu \text{m}^2 \text{s}^{-1}$) used before for rationalizing the peak in $p(K)$.

The very good agreement of equation (7) with our experimental data, based on a nonzero value of $\theta$ but also an increased value of $\alpha$, confirms the assumption that p-granule trajectories are significantly perturbed by static localization errors: nonzero static localization errors induce a constant offset in MSDs, resulting in an underestimate of the actual scaling exponent when fitting only with a simple power law (equation (2)) on short time scales. Therefore, the actual anomaly value has to be larger (here: by $\Delta \alpha \approx 0.2$) than estimated by the simple-power law fit. Consequently, the PDF $p(\alpha)$ shown in figure 2(a), found by fitting TA-MSDs with equation (2), has to be shifted to larger values by, on average, $\Delta \alpha = 0.2$, yielding an increased fraction of superdiffusive trajectories (35% instead of 14%). It is worth noting, however, that the approach of simply shifting the PDF can only serve as a rough estimate here since trajectories are not corrected individually. For sufficiently long trajectories an individual correction could be performed, for example, by a resampling approach [35]. Due to the too short trajectory lengths of p-granules, this approach did not yield meaningful results, leaving the ensemble-averaged shift as the only reasonable approach.

Let us discuss now, to which extent the perturbations by a static localization offset affect the conclusion that p-granule motion represents an FBM process. Given that all increment PDFs showed a very similar shape, irrespective of the particular value of $\alpha$ (cf figure 3), the update of $p(\alpha)$ does not necessitate additional considerations on the Gaussianity of the underlying process. Yet, the good agreement of our experimental data with the FBM prediction (equation (6)), shown in figure 2(d), appears more questionable since the VACF is very sensitive to the nature of the random walk [14].

Since static localization errors ($\theta > 0$) reduce the value of $C(\xi = 1)$ (cf equation (7)), it is tempting to assume that this change is accompanied, at least to first-order approximation, by an overall reduction of the apparent anomaly exponent by some $\Delta \alpha$, hence restoring the good overlap with equation (6). In fact, using equation (7) to plot $C(\xi = 1)$ as a function of $\alpha$ for different choices of the static localization error (set by the value of $\theta$) can, on average, be captured well by equation (6) when adjusting $\alpha$ accordingly (see
representative examples for \( n = 3 \) in figure 5(a)). The adjustment \( \Delta \alpha \) required for this, shows a moderate and monotonous increase for increasing values of \( \theta \), that can be approximated by a linear relation \( \Delta \alpha \approx \theta/10 \) in the range \( 0.5 \leq \theta \leq 2 \) (cf figure 5(b)). Therefore, the agreement of our experimental data and equation (6), as shown in figure 2(d), is due to a sufficiently small static localization error, \( \theta \approx 1.8 \), that leads to lower values of \( C(\xi = 1) \) but also to a reduction of anomaly exponents by \( \Delta \alpha \approx 0.2 \) when extracting \( \alpha \) by a simple power-law fit to MSDs, eventually restoring the overall shape of equation (6).

Although this line of argumentation at first glance only corrects erroneous conclusions drawn from an oversimplified analysis procedure, all steps explicitly rely on predictions for FBM processes (e.g. equation (7)). Thus, this correction provides even further support to the notion that p-granule motion is an FBM process.

As a final piece of evidence that p-granule motion is an FBM process, we also inspected the power spectral density (PSD) of trajectories. For FBM, the PSD is known to show a power-law decay toward the asymptotic value \( C(\xi = 1) \), extracted from equation (7) as a function of \( \alpha \) for \( n = 3 \) and varying values of the static localization offset \( \theta \) (color-coded symbols) follow overall the shape of equation (6), but with adjusted values of the anomaly exponents, i.e. \( \alpha \to \alpha + \Delta \alpha \) (full colored lines). The unshifted FBM prediction (equation (6)) is shown as grey-dashed line. Please note that for \( \theta = 0 \) no static but still a dynamic localization error is present in equation (7). In this case, somewhat stronger shape changes with respect to equation (6) are visible (red crosses versus full red line) and the shift \( \Delta \alpha \) changes sign. Hence, \( \Delta \alpha = 0 \) requires dynamic and static localization errors to cancel each other, demanding a value \( \theta > 0 \). The required correction \( \Delta \alpha \) grows monotonously with \( \theta \) and is almost linear in the range \( 0.5 \leq \theta \leq 2 \). Please note the expected crossover of \( \Delta \alpha \) from negative to positive values at a finite value of \( \theta \).

Figure 5. (a) The typical VACF values, \( C(\xi = 1) \), extracted from equation (7) as a function of \( \alpha \) for \( n = 3 \) and varying values of the static localization offset \( \theta \) (color-coded symbols) follow overall the shape of equation (6), but with adjusted values of the anomaly exponents, i.e. \( \alpha \to \alpha + \Delta \alpha \) (full colored lines). The unshifted FBM prediction (equation (6)) is shown as grey-dashed line. Please note that for \( \theta = 0 \) no static but still a dynamic localization error is present in equation (7). In this case, somewhat stronger shape changes with respect to equation (6) are visible (red crosses versus full red line) and the shift \( \Delta \alpha \) changes sign. Hence, \( \Delta \alpha = 0 \) requires dynamic and static localization errors to cancel each other, demanding a value \( \theta > 0 \). (b) The required correction \( \Delta \alpha \) grows monotonously with \( \theta \) and is almost linear in the range \( 0.5 \leq \theta \leq 2 \). Please note the expected crossover of \( \Delta \alpha \) from negative to positive values at a finite value of \( \theta \).
too much of a generalization, the found behavior of p-granules may serve as a prototypical example for FBM-based random walks in active and crowded media.

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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