Physics of Living Systems

Diffusive lensing as a mechanism of intracellular transport and compartmentalization

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Abstract

While inhomogeneous diffusivity has been identified as a ubiquitous feature of the cellular interior, its implications for particle mobility and concentration at different length scales remain largely unexplored. In this work, we use agent-based simulations of diffusion to investigate how heterogeneous diffusivity affects movement and concentration of diffusing particles. We propose that a nonequilibrium mode of membraneless compartmentalization arising from the convergence of diffusive trajectories into low-diffusive sinks, which we call “diffusive lensing,” is relevant for living systems. Our work highlights the phenomenon of diffusive lensing as a potentially key driver of mesoscale dynamics in the cytoplasm, with possible far-reaching implications for biochemical processes.

eLife assessment

The authors discuss an effect, "diffusive lensing", by which particles would accumulate in high-viscosity regions - for instance in the intracellular medium. To obtain these results, the authors rely on agent-based simulations using custom rules performed with the Ito stochastic calculus convention. The "lensing effect" discussed is a direct consequence of the choice of the Ito convention without spurious drift which has been discussed before and its adequacy for the intracellular medium is insufficiently discussed and relatively doubtful. Consequently, the relevance of the presented results for biology remain unclear and based on **incomplete** evidence.

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Introduction

Diffusion is a fundamental phenomenon of transport at scales ranging from atoms to galaxies. In cells, diffusion of individual components occurs in a complex, crowded milieu (Ellis, 2001; Luby-Phelps, 1999; van den Berg et al., 2017) that is known to exhibit position-dependent diffusivity (Berret, 2016; Garner et al., 2023; Huang et al., 2022; McLaughlin et al., 2020; Śmigiel et al., 2022; Xiang et al., 2020). Diffusion can occur within or between cellular

compartments, where concentrated components carry out chemical reactions. This rich interaction of diffusion and compartmentalization provides the context for cellular biochemistry. Diffusivity varies inversely with viscosity, a key biophysical parameter of the cytoplasm (Bausch et al., 1999; Hu et al., 2017) that dictates translational and rotational mobility of proteins and, by extension, possibly influences their activity (Huang et al., 2022; Lippincott-Schwartz et al., 2001; Pan et al., 2009). While diffusivity has been implicated in modulating or driving a range of cellular processes (Molines et al., 2022; Persson et al., 2020; Xie et al., 2022), the role of *inhomogeneous* diffusivity in shaping biochemistry by regulating biomolecular concentration and dynamics remains poorly understood. Observation of diverse instances of accumulation across scales motivates our search for uncovering how space-dependent diffusivity affects cell biology. The accumulation of small molecules within the nuclear pore, for instance, has been attributed to diffusion through a viscous region (Ma et al., 2012). At the macroscale, Chladni patterns are an example of particle concentration resulting from inhomogeneous stochastic transport coefficients (Grabec, 2017). The implications of inhomogeneous diffusivity as a nonequilibrium phenomenon occurring at time scales and length scales relevant to biology remain largely unexplored. Theoretically, more information is required to specify the problem than just the diffusion constant: different mathematical interpretations of the stochastic term in diffusion equations with a spatially inhomogeneous diffusion constant result in different physical predictions (see *Appendix* for more information). Interestingly, diverse mesoscale outcomes are also seen in the case of active biological matter (Bechinger et al., 2016; Needleman and Dogic, 2017; Yeomans, 2017), density-dependent concentration of active brownian particles (Cates and Tailleur, 2015) and size-dependent condensation kinetics in the case of *C. elegans* colony formation (Chen and Ferrell, 2021). While these phenomena focus on motile energy-expending tracers, here we emphasize the underlying space-dependency of a physical property characterizing diffusion. In particular, accumulation arising from inhomogeneous diffusivity may represent a novel mechanism of effective compartmentalization, a key activity for cells in regulating biochemical processes.

In this work, we employ agent-based modeling to explore how position-dependent diffusivity can affect the distribution of tracer particles. We show that under a set of assumptions that relate to the ambiguities intrinsic to modeling inhomogeneous diffusivity (see *Appendix*), transport due to a diffusivity gradient leads to particle trajectories being biased toward areas of lower diffusivity, leading to effective compartmentalization and the growth of concentration gradients; we call this effect “diffusive lensing,” in non-quantitative analogy to the effects on light rays of media with inhomogeneous refractive index, including refraction and the formation of caustics. Analyzing particle trajectories, we show that diffusive lensing manifests differently from homogeneous diffusion at the emergent scale. We conclude that inhomogeneous diffusivity may have diverse implications for intracellular transport, from sequestering particles to modulating where and when higher-order processes such as clustering happen, in a way that is not predictable from equivalent homogeneous-diffusivity models and could affect biochemical reactions.

Results

Inhomogeneous diffusivity drives particle accumulation

We probed the effect of inhomogeneous diffusivity on particle concentration using agent-based modeling of particle dynamics (**Fig. S1A**; see *Methods*). In our modeling, the expected macroscale behavior is dictated by the Itô interpretation of heterogeneous diffusion (see *Appendix*) (*Volpe and Wehr, 2016*). Our model was non-anticipatory in that for a modeled particle traversing an inhomogeneous diffusivity, the step size distribution was defined by the diffusivity at its *present* position. Other equally consistent interpretations (such as the entirely anticipatory “isothermal” interpretation) produce different macroscale behaviors (**Fig. S1B**). The range of physically-incompatible possibilities resulting from different interpretations is known as the Itô-

Stratonovich dilemma (Lau and Lubensky, 2007; Sokolov, 2010; Tupper and Yang, 2012; Van Kampen, 1988; Volpe and Wehr, 2016). For systems at thermal equilibrium, the isothermal convention best describes transport; however, the nonequilibrium nature of the cellular interior motivates the consideration of non-isothermal conventions; the physically appropriate convention to use depends upon microscopic parameters and timescale hierarchies not captured in a coarse-grained model of diffusion. Note that while the Itô interpretation is deployed here, it is possible to convert from one interpretation to another (Volpe and Wehr, 2016) resulting in different interpretations converging at the same physical outcome (see *Appendix*). The equation used here is distinguished from the conventional 1D diffusion equation which arises from Fick's laws and is only unambiguously true for homogeneous diffusion (characterized by constant diffusivity).

Over the course of the simulation, particles accumulated in the low-diffusivity zone (**Fig. 1A, C**), consistent with steady state closed form Itô-convention solutions (Tupper and Yang, 2012). This accumulation entailed the transient depletion of particles on the high-diffusive side of the interface. A similar accumulation was observed in a smooth diffusivity gradient (**Fig. 1B, D**). In both cases, the results from agent-based modeling were corroborated by predictions of the steady-state analytical forms derived from theory. Thus, agent-based simulations demonstrate that under the Itô convention, areas of decreased diffusivity lead to increases in the concentration of diffusing particles. We term this phenomenon "diffusive lensing."

Interaction-mediated clustering is affected by heterogenous diffusivity

Diffusive lensing is an interaction-free mode of concentrating particles that stands in contrast to a more typical paradigm of particle accumulation: interaction-driven formation of higher-order structures like protein complexes, gels, crystals and phase-separated condensates (Banani et al., 2017; Vekilov, 2010; Wu et al., 2023). How might interaction-induced clustering be modulated by inhomogeneous diffusion in a cellular context? To address this question, we heuristically modeled inter-particle interactions via a neighbor-sensing scheme in high and low interaction-strength regimes. The scheme involved using a step size for the modeled particle, which decreases as the number of particles in the vicinity increases (see *Methods*). At low interaction strength, clustering occurred only at the low-diffusivity end of a gradient (**Fig. 2A**), while the same interaction strength was insufficient to produce clusters in a uniform diffusivity distribution (**Fig. S2A, S2C**). In contrast, a high interaction strength resulted in robust clustering manifesting before particle gradient formation reached the steady state, leading to clustering towards the high-diffusivity side of the simulation region as well (**Fig. 2B**). At this high interaction strength, the clustering rate remained the same throughout the region in the absence of a gradient (**Fig. S2B, S2D**). Taken together, the results reveal that diffusive lensing can modulate clustering and under certain circumstances cause diffusivity-dependent localized cluster formation, and furthermore that the relative strengths and timescales of each phenomenon quantitatively dictate whether increased clustering will preferentially occur in low-diffusive zones. Similar density-dependent clustering is observed in the case of active Brownian particles during motility-induced phase separation (Cates and Tailleur, 2015). Effects of diffusive lensing on particle concentration may additionally regulate reaction rates and drive stochastic clustering of enzymes (Jilkine et al., 2011).

Heterogeneous diffusion alters bulk particle motion as measured by *in silico* microrheology

The diffusion coefficient is a fundamental biophysical parameter that affects numerous other phenomena, including biochemical reaction rates. To elucidate particle diffusion at the microscale in the context of diffusive lensing, we used an *in silico* implementation of microrheology to analyze particle trajectories (see *Methods*; **Fig. S3A**). We computed the mean squared

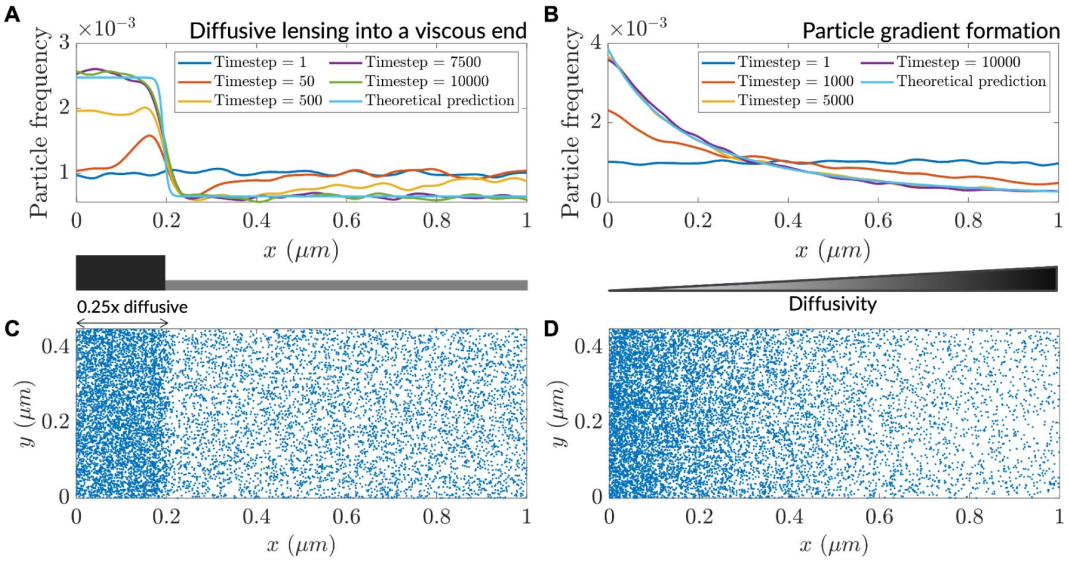

Figure 1

Low diffusivity leads to accumulation of particles.

(**A**) Particle distribution at various timesteps of a simulation with a step-like lower-diffusivity region. (**B**) Particle distribution at various timesteps for a simulation with a diffusivity gradient. (**C**) Steady-state particle distribution for the simulation in (A). (**D**) Steady-state particle distribution for the simulation in (B).

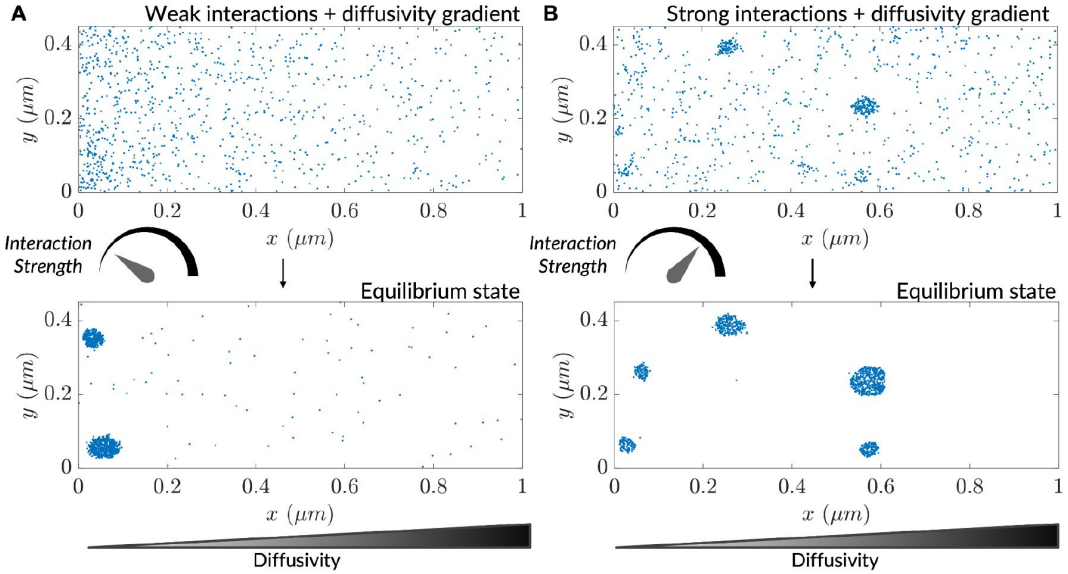

Figure 2

Interaction-driven clustering is modulated by heterogenous diffusivity.

(**A**) Progress of a simulation comprising particles possessing weak interactions (k =0.04 is the interaction strength; see *Methods*), initialized with a uniform concentration of particles. (**B**) Progress of a simulation comprising particles possessing strong interactions (k =0.1), initialized with a uniform concentration of particles.

displacements (MSDs) for uniform diffusivity simulations (in the case of unencumbered and confined diffusion) and used these to understand how MSD is affected by heterogenous diffusivity in two cases: a continuous diffusivity gradient and a discrete step in diffusivity.

Particle diffusion was unencumbered in the case of large bounds (relative to step size) (**Fig. 3A**) and confined in the case of small bounds (**Fig. 3B**) all in agreement with earlier results (Dix and Verkman, 2008; Saxton, 2007). The MSD at saturation in homogeneously diffusive systems was found to be agnostic to the underlying uniform diffusivity of the system, indicating that it is exclusively determined by the simulation region size. In contrast, particles in a diffusivity gradient exhibited dynamics intermediate to those of homogeneous high and low diffusivity cases, both in the diffusion coefficient and saturation MSD (**Fig. 3C**, inset). The lowering of the saturation MSD reflects particle diffusion occurring within apparent simulation region bounds that confine more than the actual simulation region size. We note that such effective modifications of geometry are also a general feature of optical lensing. Apparent bounds were also found to occur in the two-zone diffusivity case (as in **Fig. 1A**) where, at steady-state, particles populated the simulation region non-uniformly (**Fig. S3B**). For most of the diffusivity ratio parameter space, irrespective of whether the smaller zones were more or less diffusive relative to the bulk, a reduction in MSD was seen indicating effectively lower diffusion bounds (**Fig. 3D**). The magnitude of reduction depended on whether most particles resided in the larger or smaller of the two zones. In one observed case $(\frac{\mu_i}{\mu_o} = 0.25)$, however, the saturation MSD was higher than what was seen in the homogeneous diffusion scenario possibly due to particles robustly populating the bulk milieu followed by directed motion into the low-diffusive zone. The saturation MSD was also found to depend on the location of the low-diffusive zone: a more-centered zone resulted in a lowered saturation value, possibly due to weaker ratchet effects (**Fig. S3C, D**). These results point to the insufficiency of using the diffusion coefficient alone to describe diffusion in heterogenous milieu. They also indicate a potentially rich interplay between heterogenous diffusivity and anomalous diffusion that requires further investigation.

In silico FRAP in heterogeneously diffusive environments reveals drivers of mesoscale dynamics

The *in silico* microrheology analysis we performed provided insights into dynamics at the single-particle level (i.e., the microscale). To explore collective, emergent behaviors at the mesoscale while continuing to make contact with feasible experiments, we employed an *in silico* version of fluorescence recovery after photobleaching (*in silico* FRAP) (**Fig. S4A, B**), in more cell-like inhomogeneous environments. In particular, we modeled low-diffusive patches/granules in a cell using a three-parameter disc-packing setup comprising granule radius (r), packing density (φ) and the ratio of granule diffusivity to bulk diffusivity $(\frac{\mu_i}{\mu_o})$ (see *Methods*). We investigated the effect on dynamics of varying these parameters individually, with the goal of gaining understanding of the effects of varying the amount, nature and distribution of viscogens in cells. In all cases, the *in silico* "photobleaching" event was conducted after the steady-state was attained (**Fig. S4C,D,E**). To explain observed changes in the recovery time that would be measured in a FRAP-type experiment, we probed how the mean dwell time of particles in low-diffusive granules varies as a function of these parameters. A decrease in the diffusivity ratio $(\frac{\mu_i}{\mu_o})$ at fixed φ and r resulted in a decline in measured particle mobility, as characterized by an increase in the simulated FRAP $t_{1/2}$ values (**Fig. 4A**). Decreasing $\frac{\mu_i}{\mu_o}$ from 1 to 0.1 caused an approximate doubling of $t_{1/2}$ (or halving of diffusivity). Similar reduction in mobility was observed upon variation φ of or r separately, keeping the diffusivity ratio constant (**Fig. 4B, C**). The decrease in average mobility in all three cases arose from changes in flux between the low-diffusive and bulk zones, as reflected by an increase in mean dwell times of particles within low-diffusive granules (**Fig. S4F,G,H**). Furthermore, such reductions in mobility were emergent in that they arose from the interplay

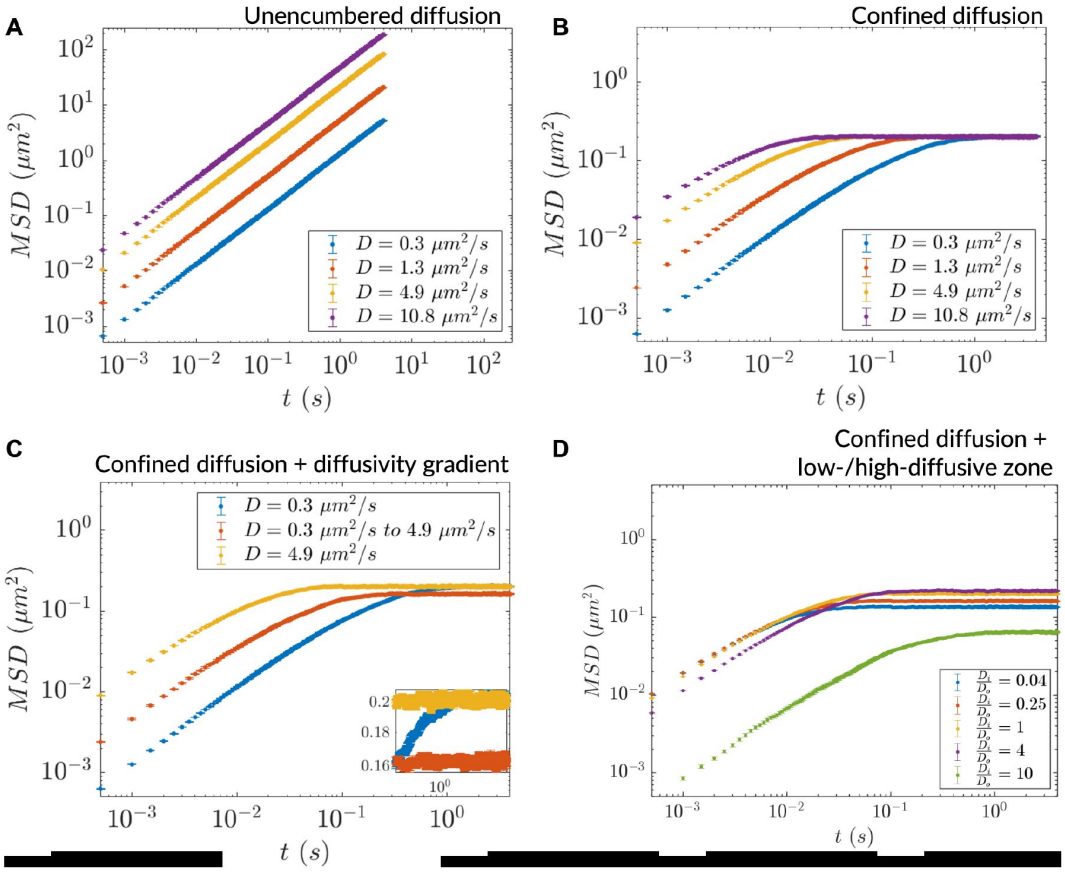

Figure 3

Heterogeneous diffusion alters bulk particle motion as measured by *in silico* microrheology.

(**A**) MSD versus time for homogeneous diffusion of 10,000 particles in a 5 mm x 5 mm simulation region. (**B**) Same as (A) for homogeneous diffusion in a more tightly bounded simulation region (1 μm x 0.45 μm). (**C**) MSD versus time for inhomogeneous diffusion in a diffusivity gradient versus homogeneous diffusion in the extreme diffusivity cases (simulation region size: 1 μm x 0.45 μm). Inset: zoomed region showing differential saturation of the MSD. (**D**) MSD versus time for inhomogeneous diffusion due to a stepwise diffusivity distribution with diffusivity ratio $\frac{\mu_i}{\mu_o}$ relative to the bulk (simulation region size: 1 μm x 0.45 μm). In all cases, n = 10,000 particles for MSD calculation (error bars denote SEM).

between granular diffusivity and bulk-granule fluxes, as the regions of interest in the simulated photobleaching events comprised granules and the surrounding bulk environment. To investigate whether particle dynamics is affected by the underlying topography realizing the system's diffusivity, we averaged the granular and bulk diffusivity values to produce weighted-average diffusivity values, and compared *in silico* recovery in these simulations to that of the equivalent granule-comprising simulations (**Fig. 4D**). We conclude that inhomogeneity in diffusivity drives rapid effective dynamics via fluxes between the granular (interior) and bulk (exterior) environments, creating "diffusive highways" for particles to move rapidly between low-diffusive regions. The diffusive lensing of particles into low-diffusive zones, and their consequent dwelling in these regions, can be tuned by modulating the underlying diffusivity distribution in myriad ways.

Discussion

The complex milieu of the cellular interior has been recently shown to feature heterogeneous diffusivity (Garner et al., 2023; Huang et al., 2022; McLaughlin et al., 2020; Parry et al., 2014; Śmigiel et al., 2022; Xiang et al., 2020), yet the consequences of such inhomogeneity on compartmentalization and mesoscale molecular dynamics have remained unclear. Through agent-based modeling of diffusion using the Itô integration convention, we show that heterogenous diffusivity can lead to simulated particle trajectories converging into low-diffusive hotspots, causing the accumulation of diffusing particles into membraneless compartments defined by the lower-diffusivity zones. We term this mode of transport "diffusive lensing". The underlying conclusions from our 2D simulations extend to 3D directly (see *Methods*). Diffusive lensing has wide-ranging effects on particle distribution and dynamics and, furthermore, it can occur across a wide parameter space. We therefore speculate that diffusive lensing is a ubiquitous phenomenon in living systems.

We found that inhomogeneous diffusivity allows for particle mobility at the microscale and mesoscale to be different from that expected in the presence of homogeneous diffusion. Such an expectation is in line with predicted and observed deviations from normal diffusion in cells (Bancaud et al., 2012; Baum et al., 2014). The relative strengths of diffusive lensing and inter-particle interactions (if any) determined the extent to which clustering was modulated by diffusive lensing: this interplay may be important for determining the effects of inhomogeneous diffusivity on biochemical reaction rates. In these simulations of clustering, particle concentration did not affect diffusivity. In the case that particle concentration decreases diffusivity (for example in the case of branched polysaccharides like glycogen), diffusive lensing may create a positive feedback loop that drives particles into areas where low diffusivity has been nucleated. The effect of diffusive lensing on runaway pathological processes like protein aggregation is a potential direction for future work.

Spatially-averaged effective diffusion timescales were found to depend on the microscopic diffusivity distribution: the same average diffusivity can give rise to slower or faster dynamics depending on whether it is realized via homogeneous or heterogenous diffusivity distributions. In the latter case, the bulk region interspersed between the low-diffusive hotspots provides "diffusive highways" that contribute to large fluxes at the diffusivity interface, thereby accounting for the faster dynamics. Such expressways and their associated fluxes may impact reaction kinetics by altering substrate turnover rates, congruent with the model of unusual transport processes potentially modifying reaction kinetics (Bénichou et al., 2010). In the context of subcellular low-diffusive regions (Garner et al., 2023), cells may compensate for geometry-imposed constraints on packing density and size of these regions by altering the diffusivity ratio (against the bulk milieu) instead. To map the detailed effects of inhomogeneous diffusivity on reaction rates, however, our work suggests that a key prerequisite is to chart a suitable set of metaparameters

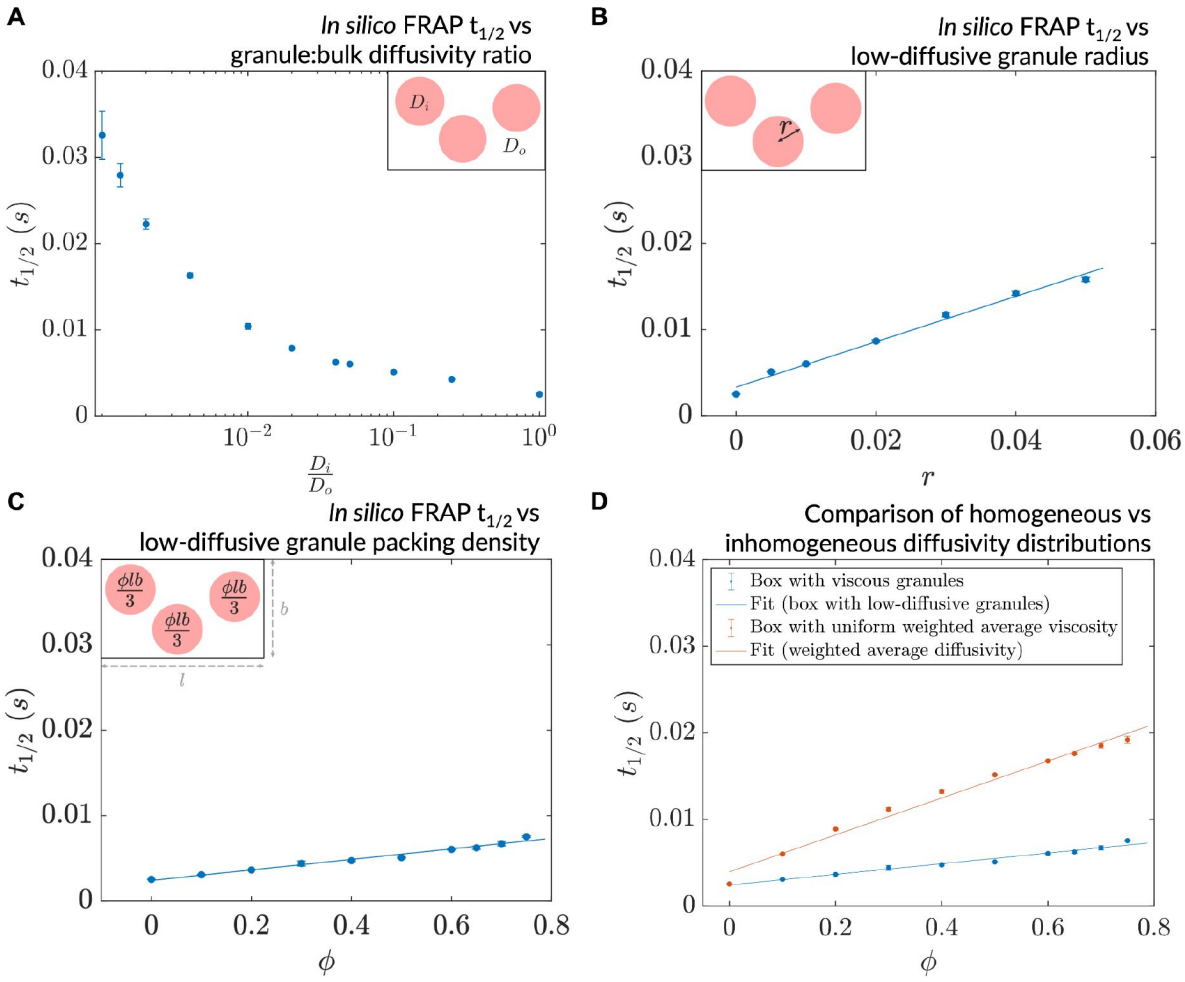

Figure 4

A decrease in granule diffusivity, an increase in granule radius, or packing density slows down mesoscale dynamics.

(**A**) Simulated FRAP $t_{1/2}$ as a function of granule:bulk diffusivity ratio (r= 0.01 μm, ϕ = 0.6). (**B**) Simulated FRAP $t_{1/2}$ as a function of granule radius $(\frac{\mu_i}{\mu_o} = 0.05,\ \phi = 0.6)$. (**C**) Simulated FRAP $t_{1/2}$ as a function of granule packing density $(\frac{\mu_i}{\mu_o} = 0.05,\ r = 0.01\ \mu m)$. (**D**) Simulated FRAP $t_{1/2}$ for homogeneous and inhomogeneous diffusivity setups realizing the same effective diffusivities $(\frac{\mu_i}{\mu_o} = 0.05,\ r = 0.01\ \mu m)$. In all cases, n = 3 ROIs were chosen for the simulated photobleaching (error bars denote SEM).

that provide an adequate description of inhomogeneous diffusion (Jin and Verkman, 2007), as a one-parameter description relying exclusively on the average diffusion coefficient is insufficient to fully specify the dynamics.

Changes in viscosity have been shown to occur in the context of cellular processes including cell death (Kuimova et al., 2008), stress adaptation (Persson et al., 2020) and protein aggregation (Thompson et al., 2015). At any given time point, intracellular transport dynamics arises emergently from contributions across length scales ranging from crowding in the bulk milieu due to proteins (Wang et al., 2010) and large biomolecules (Delarue et al., 2018) to cytoskeleton (Carlini et al., 2020; Chaubet et al., 2020) and active flows in the cytoplasm (Arcizet et al., 2008), all leading to unusual anomalous diffusive behaviors at the mesoscale (Banks and Fradin, 2005; Bressloff, 2014; Dix and Verkman, 2008; Höfling and Franosch, 2013; Kuznetsova et al., 2015; Swaminathan et al., 1997; Zhou et al., 2008). These diffusive behaviors cannot be decoupled from the intrinsic heterogeneity in biomolecular properties themselves (Heald and Cohen-Fix, 2014; Milo and Phillips, 2015). The effects of all of these subcellular determinants and energy-dependent processes on how position-dependent diffusivity is maintained in a cell remains unclear.

Not all cases of heterogeneous diffusivity will lead to diffusive lensing. This ambiguity is captured by the so-called Itô-Stratonovich dilemma (Lau and Lubensky, 2007; Sokolov, 2010; Tupper and Yang, 2012; Van Kampen, 1988; Volpe and Wehr, 2016). Any mathematical conceptualization of diffusion in the presence of position-dependent diffusivity must confront this dilemma, according to which the steady-state concentration distribution of a diffusing tracer depends not only on the localized diffusivity distribution but also on conventions based on microscopic parameters not captured in a coarse-grained model of diffusion; these parameters might, for example, include correlation lengths and times of viscogens or physical characteristics of polymers (Bo et al., 2021; Kupferman et al., 2004; Lau and Lubensky, 2007; Sokolov, 2010; Tupper and Yang, 2012; Van Kampen, 1988; Vishen et al., 2019). We speculate that any source of heterogeneity in diffusivity (including, but not limited to: mesh size experienced by the diffusing tracer, temperature changes, viscogen identity and concentration) can, in turn, modulate diffusive lensing by means of altering either the particle or the environment-induced noise relaxation time. While the Itô convention is deployed here to model the nonequilibrium cellular interior (Gnesotto et al., 2018; Phillips et al., 2012), in some cases the isothermal convention may be better suited for modeling transport. The choice of the convention (and the effect of the dilemma, by extension) may also be subverted by recasting the dynamics into an alternate convention by taking suitable drift terms into consideration (see *Appendix*). Indeed, diverse conventions have been used to model experimentally observed accumulation arising from varied sources of such position-dependent noise (Bringuier, 2011; Pesce et al., 2013; Volpe and Wehr, 2016).

Our work underscores the need to not only examine diffusivity distributions *in vivo* as a function of local composition and the environment, but also to study their time-evolution in response to external stimuli. More speculatively, we suggest that diffusive lensing serves as a potential candidate for a rudimentary mode of pre-biotic compartmentalization. Lensing-driven accumulation of diverse biomolecules may have served to produce chemically enriched spaces, acting as an antecedent of more sophisticated, membrane-bound and membraneless organizational modalities; such a protocell organization is orthogonal to currently studied models (Monnard and Walde, 2015). This work demonstrates that diffusive lensing can have strong effects on transport and may be common in cellular contexts, modulating both passive and active flows. Future experimental and theoretical work will elucidate the extent of lensing inside and outside of cells and its effects on the biochemical reactions that sustain life.

Methods

Agent-based modeling (random walk simulations)

Agent-based modeling of diffusion was conducted via 2D random walk simulations. Irrespective of how the Itô-Stratonovich dilemma is interrogated, the underlying diffusion equations contain additive, separable contributions from each dimension, and this extends to 3D as well. Calculations were, therefore, carried out in 2D for simplicity and visualizability. Non-interacting point particles were initialized uniformly in a 2D simulation region with an aspect ratio matching that of an E. coli bacterium (Phillips et al., n.d.). During each time step (also termed epoch or frame), every particle was moved along each coordinate by step sizes sampled from a uniform distribution, $\mathcal{U}(-S, S)$, where S denotes the step size limit. Across a large number of steps, the distribution of displacements converges to the normal distribution by virtue of the central limit theorem. While sampling was not performed via the normal distribution directly by using the diffusion coefficient (D) as a parameter, the diffusion coefficient was instead arrived at as an emergent property of trajectories comprising a simulation, in a ground-up fashion. Reflection off the wall was modeled using a mirror-image rule. To model a zone of differential diffusivity relative to bulk diffusivity (either a fluid or a diffusivity zone), particle step sizes were sampled from zones characterized by different diffusivities, noting that the diffusion coefficient and diffusivity are inversely related (Phillips et al., n.d.) and $S \propto \sqrt{D}$. At all times, step sizes were sampled from distributions defined by the diffusivity around the present position in accordance with the Itô interpretation of multiplicative noise (Volpe and Wehr, 2016) (for theoretical predictions of the steady-state behaviors, see *Numerical methods for the diffusion equations*). In all simulations, a set seed of 1 was used for the random number generator. Simulations were run on MATLAB R2020a on Sherlock (a high-performance computing cluster at Stanford).

In the simulations which included inter-particle interactions, these interactions were modeled via a neighbor-sensing approach. The step size limit was modified as per the relation, $S_{eff}=Se^{-kn}$, where k denotes the sensing strength and n denotes the number of neighbors (defined as those particles lying within a cutoff span around the particle in question). Such a rule-based approach modeled an effective attractive potential for the inter-particle interactions. Local density calculation used the same cutoff and the data were normalized to the mean local density of particles during initialization. Considering the computational work due to neighbor-sensing, a smaller number of particles (10^3) were deployed, for a longer period of 2×10^4epochs.

In the low-diffusive granule simulations, the granules were modeled as disks with randomly initialized centers and fixed radii (r), covering the simulation region up to a desired packing density, φ. The algorithm saturated for $\varphi \geq 0.6$, in which case, the disks were generated as per cubic close packing and their positions were incrementally deviated over 10^9 steps to reduce local ordering as much as possible. The ratio of diffusivity inside the granules to diffusivity outside the granules $\left(\frac{\mu_i}{\mu_o}\right)$ was the third parameter under consideration. No two disks were allowed to overlap and all disks were kept confined within the boundaries of the simulation region. The default setup is as follows: r = 0.01 μm (uniform), φ = 0.6 (that is, 60% of the simulation region is covered by the granules) and $\frac{\mu_i}{\mu_o} = 0.05$. Titration of one of these three parameters involved keeping the other two at the specified levels.

Numerical methods for the diffusion equations

The Fokker-Planck equations corresponding to the Ito, Stratonovich and isothermal interpretations of inhomogeneous diffusion are as follows (Gardiner, 2004; Tupper and Yang, 2012) (here $c(x,t)$ denotes the concentration distribution and $D(x)$ denotes the position-

dependent diffusivity):

$$\text{Itô interpretation: } \frac{\partial c}{\partial t} = \frac{\partial^2 cD}{\partial x^2},$$

$$\text{Stratonovich interpretation: } \frac{\partial c}{\partial t} = \frac{\partial}{\partial x}\left(\sqrt{D}\frac{\partial c\sqrt{D}}{\partial x}\right),$$

$$\text{Isothermal interpretation: } \frac{\partial c}{\partial t} = \frac{\partial}{\partial x}\left(D\frac{\partial c}{\partial x}\right).$$

These equations were numerically evaluated via forward time centered space (FTCS) schemes, with length and time increments set as $10^{-3}$and 5×10^{-7} arbitrary units, respectively, and the number of time steps was set to 10^5. A gaussian well profile was used for the diffusion coefficient and the initial condition for the concentration distribution was a uniform distribution (**Fig. S1B**). For the theoretical prediction in each case, the following relation is used: $c(x,t)D(x)^{1-\alpha}$= *constant* in steady-state, where α denotes the integration convention used (Tupper and Yang, 2012). Analysis and data visualization were performed on MATLAB R2019a.

In silico microrheology

Analysis of particle trajectories was carried out via quantifying the mean squared displacements (MSD). These were calculated from 10^4 trajectories (each 10^5 timesteps in duration) per simulation. The timestep was set as 50 μs so that the diffusion coefficient was ≈ 5 $\mu m^2/s$ (order of magnitude for a small protein's mobility in the *E. coli* cytoplasm (Milo and Phillips, 2015)).

In silico FRAP

In silico fluorescence recovery after photobleaching (FRAP) studies were performed on the diffusion simulations to quantify emergent dynamics at the mesoscale. 10^5 particles were deployed for a total duration of 0.5 s (10^4 epochs). Circular regions (radius of 0.2 μm) were chosen as the regions of interest (ROIs). *In silico* photobleaching was instantaneously performed and involved assigning the particles in the ROI the photobleach status. The background was chosen from a uniform diffusivity setup to ensure that the normalization is standardized. The outward turnover of these particles and the simultaneous inward flux of unbleached particles were captured via $t_{1/2}$, the time taken for recovery up to the 50% of the steady state level of unbleached particles in the ROI (Sprague and McNally, 2005). In these simulations, $t_{1/2}$ connotes the time taken for the number of "unbleached" particles in the ROI to reach 50% of the steady-state value. To dissect particles' behavior during the simulation (in terms of bias towards inhabiting the low-diffusive granules), we calculated the mean dwell time across all particles, per simulation. This involved averaging the periods (of any duration) spent by particles inside low-diffusive granules. For normalization, the total simulation duration was used (0.5 s).

Code Availability

Code utilized in this study is available as a Zenodo repository (*https://doi.org/10.5281/zenodo.7957931*).

Author Contributions

A.R.V., K.H.L., D.M.W., and O.B., conceptualized the project. A.R.V. performed the simulations. A.R.V., and K.H.L. conducted the analysis. A.R.V. wrote the original draft. A.R.V., K.H.L., D.M.W., and O.B., reviewed and edited the manuscript. D.M.W. and O.B. supervised the project.

Declaration of Interest

The authors declare no competing interests.

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Supplementary Figures

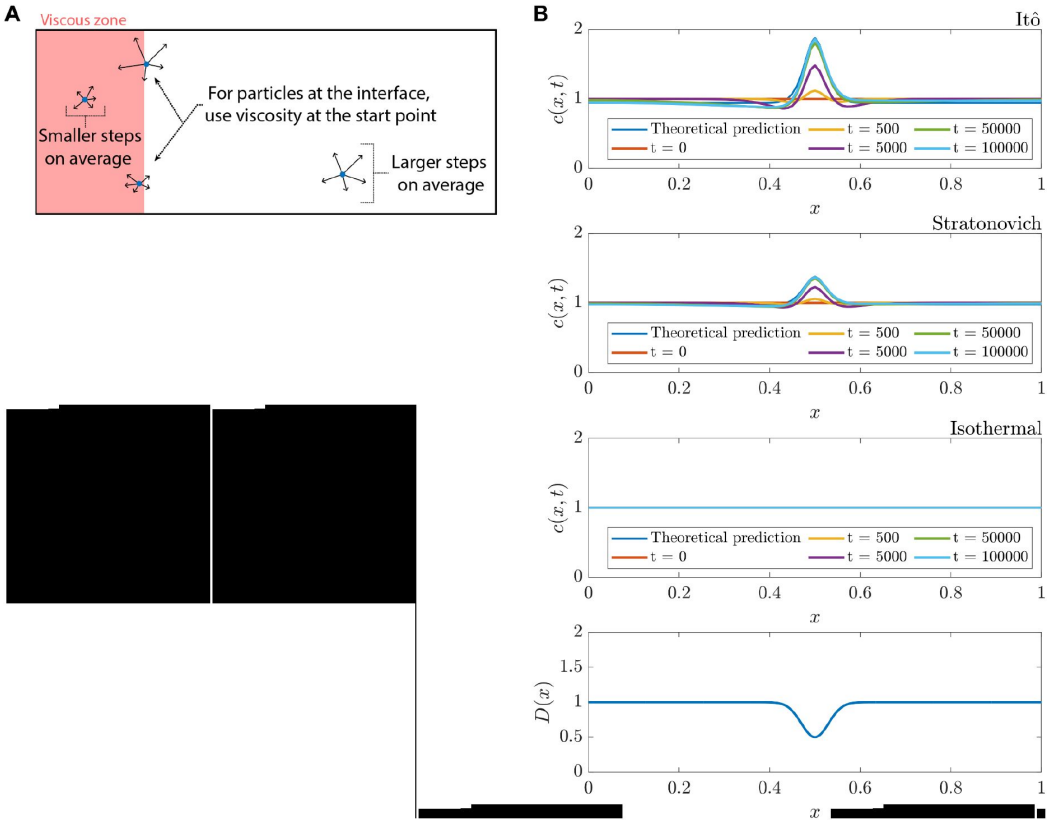


Figure S1

Itô convention leads to Fokker-Planck diffusion, contrasting canonical ("Fickian") homogenization.

(**A**) Agent-based modeling of particle dynamics used in this study. Choosing the diffusivity at the start point of a particle hop is in line with the Itô interpretation. (**B**) Numerical solutions for drift-less Fokker-Planck equations with an inhomogeneous diffusion coefficient, for the Itô, Stratonovich and isothermal conventions.

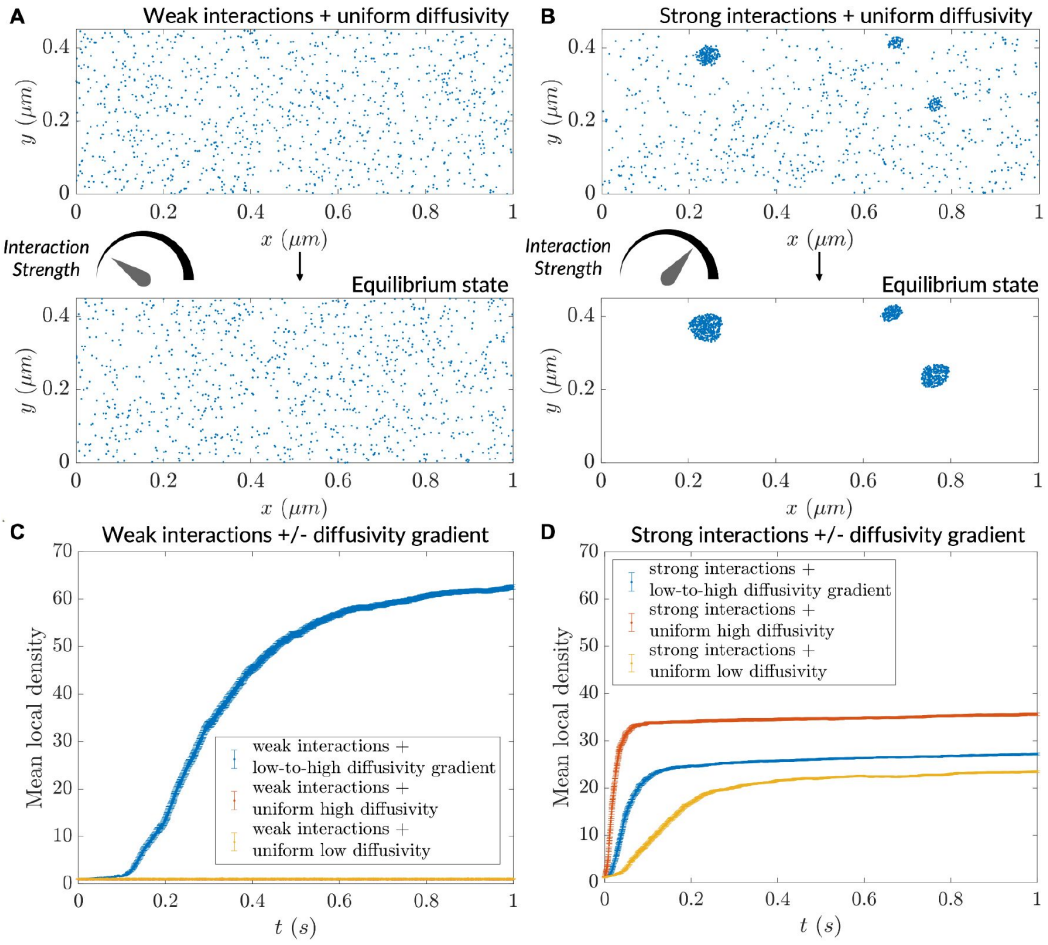


Figure S2

Particle clustering at different strengths in homogeneous versus heterogeneous diffusivity environments.

(**A**) Progress of a simulation comprising particles possessing weak interactions ($k = 0.04$), initialized with a uniform concentration of particles; no diffusivity gradient used here. (**B**) Progress of a simulation comprising particles possessing weak interactions ($k = 0.1$), initialized with a uniform concentration of particles; no diffusivity gradient used here. (**C**) Mean local density versus time for particles possessing weak interaction strength. (**D**) Mean local density versus time for particles possessing strong interaction strength. For (C) and (D), n = 1000 particles for mean local density calculation (error bars denote SEM).

Figure S3

Magnitude and distribution of inhomogeneity in diffusivity affects diffusive lensing.

(**A**) Analysis of simulation trajectories via *in silico* microrheology. (**B**) Increasing diffusiophoretic extent due to variation of the zone diffusivity in a chamber comprising a low-diffusive end. (**C**) Mean squared displacement after transition to normal diffusion (saturation MSD) depends both on the magnitude of diffusivity difference and the location of the zone itself. n = 10,000 particles for MSD calculation (error bars denote SEM). (**D**) Histogram of $\|X\|^2$ at the end of the run for the cases of the 0.25x low-diffusive zone located at the simulation region edge versus the center.

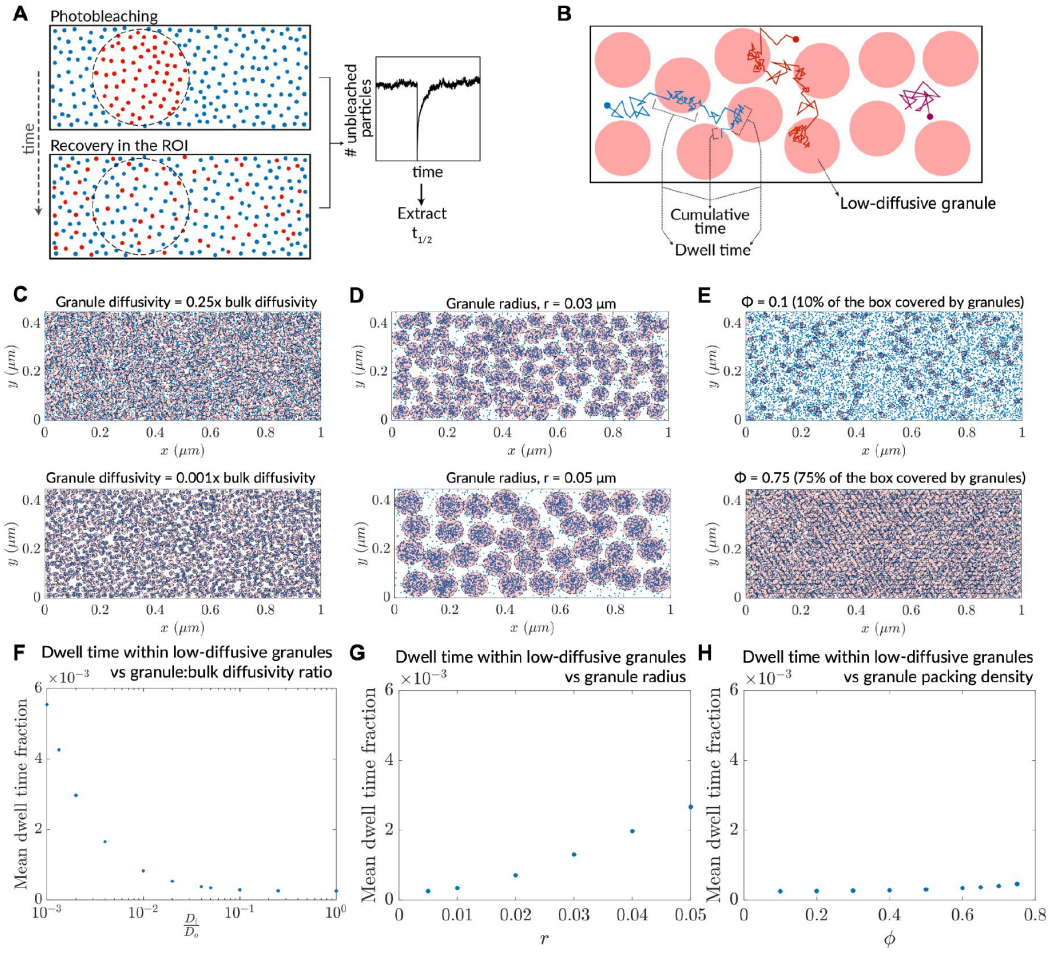


Figure S4

Dwell times for particles in low-diffusive granules dictate FRAP kinetics.

(**A**) The *in silico* implementation of FRAP used in this study. (**B**) Methodology for determining mean dwell time of particles in low-diffusive granules, from a set of simulation trajectories. (**C**) Steady-states of systems in the variation of granule diffusivity, before commencing *in silico* FRAP. (**D**) Steady-states of systems in the variation of granule radius, before commencing *in silico* FRAP. (**E**) Steady-states of systems in the variation of granule packing density, before commencing *in silico* FRAP. (**F**) Mean dwell time fraction variation as a function of granule:bulk diffusivity. (**G**) Mean dwell time fraction variation as a function of granule radius. (**H**) Mean dwell time fraction variation as a function of granule packing density. For (F)-(H), n = 10,000 particles used for calculation (error bars denote SEM).

Appendix

Itô-Stratonovich dilemma and derivation of the generalized flux for heterogeneous diffusion

The Itô-Stratonovich dilemma is an ambiguity which arises directly from any analytical attempt to solve a stochastic differential equation comprising multiplicative (or position-dependent) noise (Amir, 2020; Gardiner, 2004; Van Kampen, 1988; Volpe and Wehr, 2016). The underlying mathematical reason for the ambiguity is the non-differentiable nature of the stochastic term, which causes different Riemann sum conventions to give quantitatively different results (Gardiner, 2004). From a more physical point of view, the different conventions correspond to different hierarchies of "small" scales (correlation times and lengths of driving terms or viscogens, for example; see (Pesce et al., 2013) for a discussion of competing timescales, (Sokolov, 2010) for arriving at a context-dependent choice of interpretation). While the dilemma exists at the level of choosing an interpretation, it does not negate the appearance of diffusive lensing; indeed, only the magnitude of lensing is altered by the choice of the interpretation. This, too, may be subverted by adding an appropriate drift term to recast a stochastic differential equation (SDE) into one abiding by an alternate convention, as detailed later in this section. In summary, even the effect of the convention on the magnitude of diffusive lensing may be altered by recasting the SDE.

Formally, the dilemma can be boiled down to a choice of the parameter, α ($0 \leq \alpha \leq 1$), in a diffusion equation like $\frac{\partial c}{\partial t} = -\frac{\partial J}{\partial x} = \frac{\partial}{\partial x}\left[(1-\alpha)c\frac{dD}{dx} + D\frac{\partial c}{\partial x}\right]$, where $c(x,t)$ denotes the concentration distribution and $D(x)$ denotes the position-dependent diffusivity (see below for the derivation of the generalized flux). Clearly, for a position-dependent diffusivity, different values of α will give rise to different physical predictions. In thermal equilibrium, α must be equal to 1, but away from equilibrium it can take on different values depending on microscopic details of the physical system (Gardiner, 2004; Kupferman et al., 2004; Pesce et al., 2013; Smythe et al., 1983; Turelli, 1977; Vishen et al., 2019; Wang et al., 2020). Living cells are inherently removed from equilibrium by energy-driven processes breaking detailed balance (Gnesotto et al., 2018; Phillips et al., 2012). This motivates the calculations in this work, which use the Itô ($\alpha = 0$) integration convention, but in fact qualitatively similar results would be observed for any value of α other than 1. Taken together with the ability to recast an SDE into one confining to an alternate convention, the dilemma does not take away from our principal thesis on how low diffusivity zones can accrete particles.

Consider a diffusing tracer whose trajectory is specified by the stochastic process, $X(t)$. The stochastic process is continuous but is nowhere differentiable: the simplistic case of such a mathematical object is the Wiener process $W(t)$, also known as Brownian motion (Gardiner, 2004). Note that in general, however, the stochastic process is characterized by a deterministic drift $a(x, t)$ and diffusion $b(x, t)$. Indeed, $X(t)$ is in fact the solution of the stochastic differential equation (SDE): $dX(t) = a(x, t)dt + b(x, t)dW(t)$, where $dW(t) = \eta(t)dt$ denotes the Wiener increment and $\eta(t)$ is the Gaussian white noise.

The stochastic differential equation here comprises multiplicative noise: that is, a position-dependent function, $b(x, t)$, factors into the noise term of the SDE. This necessitates confronting the Itô-Stratonovich dilemma; we consider a general convention $\alpha \in [0, 1]$, where $\alpha = 0, 0.5, 1$ correspond to the Itô, Stratonovich and isothermal conventions respectively.

The SDE detailed above corresponds to a Fokker-Planck equation describing the time-evolution of the probability distribution of the tracer,

$$p(x,t):\ \frac{\partial p(x,t)}{\partial t} = -\frac{\partial}{\partial x}\left(a(x,t)p(x,t) + \alpha b(x,t)\frac{\partial b(x,t)}{\partial x}p(x,t)\right) + \frac{1}{2}\frac{\partial^2}{\partial x^2}\left[b^2(x,t)p(x,t)\right]$$

(substituting for α yields specific cases; see Eqn. 4.3.20 in (Gardiner, 2004), Eqns. 2.2.25, 2.2.26 in (Bressloff, 2014), Table 2 and Discussion in (Tupper and Yang, 2012)). Setting $a(x, t)$ (drift) to zero, and $b^2(x, t)$ (diffusion) to $2D$, a constant, yields Brownian motion, i.e., the canonical 1D diffusion equation which may also be derived from Fick's laws. Keeping drift as zero, but setting diffusion to $2D(x)$ (space-dependent) yields:

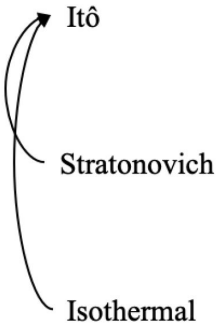
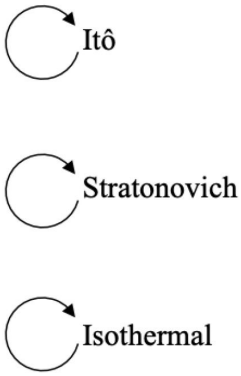
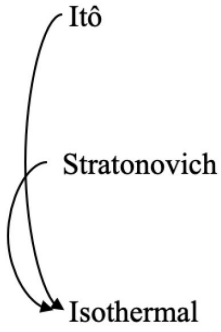
$\frac{\partial p(x,t)}{\partial t} = -\frac{\partial}{\partial x}\left(\alpha\frac{dD(x)}{dx}p(x,t)\right) + \frac{\partial^2}{\partial x^2}\left[D(x)p(x,t)\right]$, which describes our scenarios in question. This equation can be simplified as follows:

$$\frac{\partial p(x,t)}{\partial t} = -\frac{\partial}{\partial x}\left[\alpha\frac{dD(x)}{dx}p(x,t) - \frac{\partial}{\partial x}\left(D(x)p(x,t)\right)\right],$$

$$\Rightarrow \frac{\partial p(x,t)}{\partial t} = -\frac{\partial}{\partial x}\left[-(1-\alpha)p(x,t)\frac{dD(x)}{dx} - D(x)\frac{\partial p(x,t)}{\partial x}\right] = -\frac{\partial}{\partial x}J(x,t),$$

where $J = -(1-\alpha)p(x,t)\frac{dD(x)}{dx} - D(x)\frac{\partial p(x,t)}{\partial x}$ is the generalized flux. Re-writing in terms of concentration yields the final expression, $J = -(1-\alpha)c\frac{dD}{dx} - D\frac{\partial c}{\partial x}$. Substituting for α and calculating $-\frac{\partial}{\partial x}J(x,t)$ yields the equations deployed in *Methods:Numerical methods for the diffusion equations*.

Note that it is also possible to convert between different interpretations of the multiplicative noise term by adding suitable drift terms as detailed in (Volpe and Wehr 2016; Tupper and Yang 2012). In cases where thermal equilibrium is relevant, the Itô- and Stratonovich-conforming equations may be modified by adding drift terms to conform to that of the isothermal interpretation. Conversely, it is also possible to convert an equation based upon the isothermal convention to an Itô- or Stratonovich-conforming equation by modifying the drift term in the latter. Potential use cases as well as the consequences of following such conversions are detailed in these three example prescriptions below:

		
Active noise/memory effects/other sources of nonequilibrium behaviors	Nonequilibrium in case of Itô and Stratonovich, equilibrium in case of isothermal	Consistent with equilibrium; thermal noise dominates
Diffusive lensing occurs	Diffusive lensing is seen in the Itô and Stratonovich cases (Fig. S1B)	Diffusive lensing does not occur

References

Amir A. (2020) **Thinking Probabilistically: Stochastic Processes, Disordered Systems, and Their Applications**

Arcizet D, Meier B, Sackmann E, Rädler JO, Heinrich D. (2008) **Temporal analysis of active and passive transport in living cells** *Phys Rev Lett* **101**

Banani SF, Lee HO, Hyman AA, Rosen MK (2017) **Biomolecular condensates: organizers of cellular biochemistry |** *Nature Reviews Molecular Cell Biology* https://doi.org/10.1038/nrm.2017.7

Bancaud A, Lavelle C, Huet S, Ellenberg J. (2012) **A fractal model for nuclear organization: current evidence and biological implications** *Nucleic Acids Res* **40**:8783–8792

Banks DS, Fradin C. (2005) **Anomalous diffusion of proteins due to molecular crowding** *Biophys J* **89**:2960–2971

Baum M, Erdel F, Wachsmuth M, Rippe K. (2014) **Retrieving the intracellular topology from multi-scale protein mobility mapping in living cells** *Nat Commun* **5**

Bausch AR, Möller W, Sackmann E. (1999) **Measurement of local viscoelasticity and forces in living cells by magnetic tweezers** *Biophys J* **76**:573–579

Bechinger C, Di Leonardo R, Löwen H, Reichhardt C, Volpe G, Volpe G. (2016) **Active particles in complex and crowded environments** *Rev Mod Phys* **88**

Bénichou O, Chevalier C, Klafter J, Meyer B, Voituriez R. (2010) **Geometry-controlled kinetics** *Nat Chem* **2**:472–477

Berret J-F. (2016) **Local viscoelasticity of living cells measured by rotational magnetic spectroscopy** *Nat Commun* **7**

Bo S, Hubatsch L, Bauermann J, Weber CA, Jülicher F. (2021) **Stochastic dynamics of single molecules across phase boundaries** *Phys Rev Research* **3**

Bressloff PC (2014) **Stochastic Processes in Cell Biology** *Interdisciplinary Applied Mathematics*

Bringuier E. (2011) **Particle diffusion in an inhomogeneous medium** *Eur J Phys* **32**:975–992

Carlini L, Brittingham GP, Holt LJ, Kapoor TM (2020) **Microtubules Enhance Mesoscale Effective Diffusivity in the Crowded Metaphase Cytoplasm** *Dev Cell* **54**:574–582

Cates ME, Tailleur J. (2015) **Cates ME, Tailleur J. 2015. Motility-Induced Phase Separation. doi:10.1146/annurev-conmatphys-031214-014710** https://doi.org/10.1146/annurev-conmatphys-031214-014710

Chaubet L, Chaudhary AR, Heris HK, Ehrlicher AJ, Hendricks AG (2020) **Dynamic actin cross-linking governs the cytoplasm's transition to fluid-like behavior** *Mol Biol Cell* **31**:1744–1752

Chen Y, Ferrell JE (2021) **C. elegans colony formation as a condensation phenomenon** *Nat Commun* **12**

Delarue M *et al.* (2018) **mTORC1 Controls Phase Separation and the Biophysical Properties of the Cytoplasm by Tuning Crowding** *Cell* **174**:338–349

Dix JA, Verkman AS (2008) **Crowding effects on diffusion in solutions and cells** *Annu Rev Biophys* **37**:247–263

Ellis RJ (2001) **Macromolecular crowding: obvious but underappreciated** *Trends Biochem Sci* **26**:597–604

Gardiner CW (2004) **Handbook of Stochastic Methods for Physics** *Chemistry and the Natural Sciences*

Garner RM, Molines AT, Theriot JA, Chang F. (2023) **Vast heterogeneity in cytoplasmic diffusion rates revealed by nanorheology and Doppelgänger simulations** *Biophys J* https://doi.org/10.1016/j.bpj.2023.01.040

Gnesotto FS, Mura F, Gladrow J, Broedersz CP (2018) **Broken detailed balance and non-equilibrium dynamics in living systems: a review** *Rep Prog Phys* **81**

Grabec I. (2017) **Vibration driven random walk in a Chladni experiment** *Phys Lett A* **381**:59–64

Heald R, Cohen-Fix O. (2014) **Morphology and function of membrane-bound organelles** *Curr Opin Cell Biol* **26**:79–86

Höfling F, Franosch T. (2013) **Anomalous transport in the crowded world of biological cells** *Rep Prog Phys* **76**

Huang WYC, Cheng X, Ferrell JE (2022) **Cytoplasmic organization promotes protein diffusion in Xenopus extracts** *Nat Commun* **13**

Hu J, Jafari S, Han Y, Grodzinsky AJ, Cai S, Guo M. (2017) **Size- and speed-dependent mechanical behavior in living mammalian cytoplasm** *Proc Natl Acad Sci U S A* **114**:9529–9534

Jilkine A, Angenent SB, Wu LF, Altschuler SJ (2011) **A density-dependent switch drives stochastic clustering and polarization of signaling molecules** *PLoS Comput Biol* **7**

Jin S, Verkman AS (2007) **Single particle tracking of complex diffusion in membranes: simulation and detection of barrier, raft, and interaction phenomena** *J Phys Chem B* **111**:3625–3632

Kuimova MK, Yahioglu G, Levitt JA, Suhling K. (2008) **Molecular rotor measures viscosity of live cells via fluorescence lifetime imaging** *J Am Chem Soc* **130**:6672–6673

Kupferman R, Pavliotis GA, Stuart AM (2004) **It\^o versus Stratonovich white-noise limits for systems with inertia and colored multiplicative noise** *Phys Rev E* **70**

Kuznetsova IM, Zaslavsky BY, Breydo L, Turoverov KK, Uversky VN (2015) **Beyond the excluded volume effects: mechanistic complexity of the crowded milieu** *Molecules* **20**:1377–1409

Lau AWC, Lubensky TC (2007) **State-dependent diffusion: Thermodynamic consistency and its path integral formulation** *Phys Rev E* **76**

Lippincott-Schwartz J, Snapp E, Kenworthy A. (2001) **Studying protein dynamics in living cells** *Nat Rev Mol Cell Biol* **2**:444–456

Luby-Phelps K., Walter H, Brooks DE, Srere PA (1999) **Cytoarchitecture and Physical Properties of Cytoplasm: Volume, Viscosity, Diffusion, Intracellular Surface Area** *International Review of Cytology, Microcompartmentation and Phase Separation in Cytoplasm* :189–221

Ma J, Goryaynov A, Sarma A, Yang W. (2012) **Self-regulated viscous channel in the nuclear pore complex** *Proc Natl Acad Sci U S A* **109**:7326–7331

McLaughlin GA, Langdon EM, Crutchley JM, Holt LJ, Forest MG, Newby JM, Gladfelter AS (2020) **Spatial heterogeneity of the cytosol revealed by machine learning-based 3D particle tracking** *Mol Biol Cell* **31**:1498–1511

Milo R, Phillips R. (2015) **Cell Biology by the Numbers** *Garland Science*

Molines AT *et al.* (2022) **Physical properties of the cytoplasm modulate the rates of microtubule polymerization and depolymerization** *Dev Cell* **57**:466–479

Monnard P-A, Walde P. (2015) **Current Ideas about Prebiological Compartmentalization** *Life* **5**:1239–1263

Needleman D, Dogic Z. (2017) **Active matter at the interface between materials science and cell biology** *Nature Reviews Materials* **2**:1–14

Pan W, Filobelo L, Pham NDQ, Galkin O, Uzunova VV, Vekilov PG (2009) **Viscoelasticity in homogeneous protein solutions** *Phys Rev Lett* **102**

Parry BR, Surovtsev IV, Cabeen MT, O'Hern CS, Dufresne ER, Jacobs-Wagner C. (2014) **The bacterial cytoplasm has glass-like properties and is fluidized by metabolic activity** *Cell* **156**:183–194

Persson LB, Ambati VS, Brandman O. (2020) **Cellular Control of Viscosity Counters Changes in Temperature and Energy Availability** *Cell* **183**:1572–1585

Pesce G, McDaniel A, Hottovy S, Wehr J, Volpe G. (2013) **Stratonovich-to-Itô transition in noisy systems with multiplicative feedback** *Nat Commun* **4**

Phillips R, Kondev J, Theriot J, Garcia HG, Orme N. (2012) **Physical biology of the cell** *Garland Science*

Phillips R, Kondev J, Theriot J, Garcia HG, Orme N., physical-biology-cell-rob-phillips-jane-kondev-julie-theriot-hernan-garcia-nigel-orme **Phillips R, Kondev J, Theriot J, Garcia HG, Orme N. n.d. Physical biology of the cell. doi:10.1201/9781134111589/ physical-biology-cell-rob-phillips-jane-kondev-julie-theriot-hernan-garcia-nigel-orme** https://doi.org/10.1201/9781134111589/

Saxton MJ (2007) **A biological interpretation of transient anomalous subdiffusion. I. Qualitative model** *Biophys J* **92**:1178–1191

Smigiel WM, Mantovanelli L, Linnik DS, Punter M, Silberberg J, Xiang L, Xu K, Poolman B. (2022) **Protein diffusion in Escherichia coli cytoplasm scales with the mass of the complexes and is location dependent** *Science Advances* https://doi.org/10.1126/sciadv.abo5387

Smythe J, Moss F, McClintock PVE (1983) **Observation of a Noise-Induced Phase Transition with an Analog Simulator** *Phys Rev Lett* **51**:1062–1065

Sokolov IM (2010) **Itô, Stratonovich, Hänggi and all the rest: The thermodynamics of interpretation** *Chem Phys, Stochastic processes in Physics and Chemistry (in honor of Peter Hänggi)* **375**:359–363

Sprague BL, McNally JG (2005) **FRAP analysis of binding: proper and fitting** *Trends Cell Biol* **15**:84–91

Swaminathan R, Hoang CP, Verkman AS (1997) **Photobleaching recovery and anisotropy decay of green fluorescent protein GFP-S65T in solution and cells: cytoplasmic viscosity probed by green fluorescent protein translational and rotational diffusion** *Biophys J* **72**:1900–1907

Thompson AJ, Herling TW, Kubánková M, Vyšniauskas A, Knowles TPJ, Kuimova MK (2015) **Molecular Rotors Provide Insights into Microscopic Structural Changes During Protein Aggregation** *J Phys Chem B* **119**:10170–10179

Tupper PF, Yang X. (2012) **A paradox of state-dependent diffusion and how to resolve it** *Proceedings of the Royal Society A: Mathematical, Physical and Engineering Sciences* **468**:3864–3881

Turelli M. (1977) **Random environments and stochastic calculus** *Theor Popul Biol* **12**:140–178

van den Berg J, Boersma AJ, Poolman B. (2017) **Microorganisms maintain crowding homeostasis** *Nat Rev Microbiol* **15**:309–318

Van Kampen NG (1988) **Diffusion in inhomogeneous media** *J Phys Chem Solids* **49**:673–677

Vekilov PG (2010) **Phase transitions of folded proteins** *Soft Matter* **6**:5254–5272

Vishen AS, Prost J, Rao M. (2019) **Breakdown of effective temperature, power law interactions, and self-propulsion in a momentum-conserving active fluid** *Phys Rev E* **100**

Volpe G, Wehr J. (2016) **Effective drifts in dynamical systems with multiplicative noise: a review of recent progress** *Rep Prog Phys* **79**

Wang W, Cherstvy AG, Liu X, Metzler R. (2020) **Anomalous diffusion and nonergodicity for heterogeneous diffusion processes with fractional Gaussian noise** *Phys Rev E* **102**

Wang Y, Li C, Pielak GJ (2010) **Effects of proteins on protein diffusion** *J Am Chem Soc* **132**:9392–9397

Wu T, King MR, Farag M, Pappu RV, Lew MD (2023) **Wu T, King MR, Farag M, Pappu RV, Lew MD. 2023. Single fluorogen imaging reveals spatial inhomogeneities within biomolecular condensates. doi:10.1101/2023.01.26.525727** https://doi.org/10.1101/2023.01.26.525727

Xiang L, Chen K, Yan R, Li W, Xu K. (2020) **Single-molecule displacement mapping unveils nanoscale heterogeneities in intracellular diffusivity** *Nat Methods* **17**:524–530

Xie J, Najafi J, Le Borgne R, Verbavatz J-M, Durieu C, Sallé J, Minc N. (2022) **Contribution of cytoplasm viscoelastic properties to mitotic spindle positioning** *Proc Natl Acad Sci U S A* **119** https://doi.org/10.1073/pnas.2115593119

Yeomans JM (2017) **Nature's engines: active matter** *Europhys News* **48**:21–25

Zhou H-X, Rivas G, Minton AP (2008) **Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences** *Annu Rev Biophys* **37**:375–397

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Reviewer #1 (Public Review):

The revised manuscript "Diffusive lensing as a mechanism of intracellular transport and compartmentalization" is very similar to the original manuscript. The main difference between the revised and the original manuscript is that the authors have removed the reference to viscosity gradient and instead talk of diffusivity gradient. With this change the manuscript the analysis and claims in the manuscript are much more aligned. The manuscript, as the original version, explores the role of spatially varying diffusion constant in three scenarios:

(i) Spatial localization of non-particles
(ii) Clustering in presence of inter-particle interactions
(iii) Moment analysis for non-interacting particles in space with discrete patches of inhomogeneous diffusivity.

Since the manuscript has not changed much the strengths and weaknesses, in my opinion, remain similar to that of the original manuscript.

Strengths: The implications of a heterogeneous environment on phase separation and reaction kinetics in cells are under-explored. This makes the general theme of this manuscript relevant and interesting.

Weaknesses: The central part of the paper "diffusive lensing", i.e., particles localizing in the region of low diffusion constant is not new. Some of the papers authors cite already show that. The parts on phase separation and frap analysis that could provide new results are not rigorous enough for a theory paper.

I reiterate some of my comments from the original version that are valid for the revised version as well.

My main criticism was not to say that some convention should be used or some not. But instead, the main point was to say that just because there is spatial diffusion constant that does not mean there will be a spatial gradient of particles. From the authors response to my comments, it is clear that they understand the subtilties around it and are aware of the relevant papers. However, a reader not familiar with this discussion may work under the impression that if there if there is a spatialy varying diffusion constant in cell there will be an

accumulation of particles in the region of low diffusivity but that may not always be the case. Moreover, localisation of particles in the region of low diffusivity has been reported in many different context. Some of the papers that the author cite already show that. For example, in Rupprecht et al. 2018 non-isothermal interpretation is applied to the dynamics of objects inside cells.

Given that the central result is not new. The paper could still be of general interest to the biophysics community if the follow up sections (ii) Clustering in presence of inter-particle interactions and (iii) Moment analysis for non-interacting particles in space with discrete patches of inhomogeneous diffusivity were analysed rigorously.

https://doi.org/10.7554/eLife.89794.2.sa1

Reviewer #2 (Public Review):

Summary:

The authors study through theory and simulations the diffusion of microscopic particles, and aim to account for the effects of inhomogeneous viscosity and diffusion - in particular regarding the intracellular environment. They propose a mechanism, termed "Diffusive lensing", by which particles are attracted towards low-diffusivity regions where they remain trapped. To obtain these results, the authors rely on agent-based simulations using custom rules performed within the Ito stochastic calculus convention, without drift. They acknowledge the fact that this convention does not describe equilibrium systems, and that their results would not hold at equilibrium - and discard these facts by invoking the facts that cells are out-of-equilibrium. Finally, they show some applications of their findings, in particular enhanced clustering in the low-diffusivity regions. The authors conclude that as inhomogeneous diffusion is ubiquitous in life, so must their mechanism be, and hence it must be important.

Strengths:

The article is well-written, clearly intelligible, its hypotheses are stated relatively clearly and the models and mathematical derivations are compatible with these hypotheses. In the appendices, the authors connect their findings to known results for classic stochastic differential equation formalisms.

Weaknesses:

This study is, in my opinion, deeply flawed. The main problem lies in the hypotheses, in particular the choice of considering drift-less dynamics in the Ito convention. It is regrettable that the authors choose to use agent-based custom simulations with little physical motivation, rather than a well-established stochastic differential equations framework.

Indeed, stochastic conventions are a notoriously tricky business, but they are both mathematically and physically well-understood and do not result in any "dilemma" [some citations in the article, such as (Lau and Lubensky) and (Volpe and Wehr), make an unambiguous resolution of these]. In the continuous-time limit, conventions are not an intrinsic, fixed property of a system, but a choice of writing; however, whenever going from one to another, one must include a corresponding "spurious drift" that compensates the effect of this change - a mathematical subtlety that is omitted in the article (except in a quick note in the appendix): in the presence of diffusive gradients, if the drift is zero in one convention, it will thus be non-zero in another. It is well established that for equilibrium systems obeying fluctuation-dissipation, the spurious drift vanishes in the anti-Ito stochastic convention; more precisely one can write in the anti-Ito convention

dx/dt = - D(x)/kT grad U(x) + sqrt(2D(x)) dW

with D(x) the diffusion, kT the thermal energy (which is space-independent at equilibrium), and dW a d-dimensional Wiener process. Equivalently one can write in the Ito convention:

dx/dt = - D(x)/kT grad U(x) + sqrt(2D(x)) dW + div D(x) (*)

where the latter term is the spurious drift arising from convention change. This ensures that the diffusion gradients do not induce currents and probability gradients, and thus that the steady-state PDF is the Gibbs measure (this form has been confirmed experimentally, for instance, for colloidal particles near walls, that have strong diffusivity gradients despite not having significant forces). It generalizes to near-equilibrium systems with non-conservative forces and/or temperature gradient in the form:

dx/dt = F(x) + sqrt(2D(x)) dW + div D(x) (**)

where the drift field F(x) encodes these forces. In some cases, it has been shown through careful microscopic analysis that one can have effectively a different form for the last term, namely

dx/dt = F(x) + sqrt(2D(x)) dW + alpha div D(x)

where alpha is a "convention parameter" that would be =1 at equilibrium. For instance, in the Volpe and Wehr review this can occur through memory effects in robotic dynamics, or through strong fluctuation-dissipation breakdown. In a near-equilibrium system, this should be strongly justified, as the continuous-time dynamics with alpha \neq 1 and drift F would be indistinguishable from one with alpha = 1 and drift F + (1-alpha) div D: the authors would have the burden of proving that the observed (absence of) drift is indeed due to alpha\neq 1, rather than to much more common force fields F(x).

Here, without further motivation than the statement that cells are out-of-equilibrium, drifts are arbitrarily set to zero in the Ito convention, which is in (**) the equivalent to adding a force with drift $-div D$ exactly compensating the spurious drift. It is the effects of this arbitrary force that are studied in the article. The fact that it results in probability gradients is trivial once formulated this way (and in no way is this new - many of the references, for instance Volpe and Wehr, mention this). Enhanced clustering is also a trivial effect of this probability gradient (the local concentration is increased by this force field, so phase separation can occur). As a side note the "neighbor sensing" scheme to describe interactions is itself very peculiar and not physically motivated - it violates stochastic thermodynamics laws too, as detailed balance is apparently not respected. There again, the authors have chosen to disregard a century of stochastic thermodynamics in favor of a non-justified unphysical custom rule.

The authors make no further justification of their choice of driftless Ito simulations than the fact that cells are out-of-equilibrium, leaving the feeling that this is a detail. They make mentions of systems (eg glycogen, prebiotic environment) for which (near-)equilibrium physics should mostly prevail, and of fluctuation dissipation ("Diffusivity varies inversely with viscosity", in the introduction). Yet the "phenomenon" they discuss is entirely reliant on an undiscussed mechanism by which these assumptions would be completely violated (the citations they make for this - Gnesotto '18 and Phillips '12 - are simply discussions of the fact that cells are out-of-equilibrium, not on any consequences on the convention).

Finally, while inhomogeneous diffusion is ubiquitous, the strength of this effect in realistic conditions is not discussed. Even in the most "optimistic" case where alpha=0 would make sense (knowing that in the cellular context we are discussing thermal systems immersed in water and if energy consumption and metabolism were stopped alpha would relax back to 1),

the equation (*) above shows that having zero ito drift is equivalent to having a potential countering the spurious drift, with value

U(x) = kT log(D(x) / D0)

[I have assumed isotropic diffusion for simplicity here, so the div is replaced by a grad]. This means that the diffusion contrasts logarithmically compare to the chemical potential ones -- for instance a major diffusion difference of 100x is equivalent to 4.6kT in potential energy, a relatively modest effect. To prove that the authors' effect of "diffusive lensing" is involved in such a system, one would thus have to

1. observe strong spatial variations of the diffusion coefficient (this is doable, and was done before), AND
2. show that there is an enrichment of the diffusing species in the low-diffusion region inversely proportional to the diffusion, AND
3. show that this enrichment cannot be attributed to mild differences in potential energy, for instance by showing that if nonequilibrium energy consumption stops, the concentration fully homogenizes while the diffusion gradients remain.

If the authors were to successfully show all that in an experimental system, or design a theoretical framework where these effects convincingly emerge from physically realistic microscopic dynamical rules, they would have indeed discovered a new phenomenon. In contrast, the current article only demonstrates the well-known fact that when using arbitrary dynamical rules in heterogeneous diffusion simulations, one can get concentration gradients.

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Author response:

The following is the authors' response to the original reviews.

> ***eLife assessment***
>
> *The authors discuss an effect, "diffusive lensing", by which particles would accumulate in high-viscosity regions, for instance in the intracellular medium. To obtain these results, the authors rely on agent-based simulations using custom rules performed with the Ito stochastic calculus convention. The "lensing effect" discussed is a direct consequence of the choice of the Ito convention without spurious drift which has been discussed before and is likely to be inadequate for the intracellular medium, causing the presented results to likely have little relevance for biology.*

We thank the editors and the reviewers for their consideration of our manuscript. We argue in this rebuttal and revision that our results and conclusions are in fact likely to have relevance for biology. While we use the Itô convention for ease of modeling considering its non-anticipatory nature upon discretization (see (Volpe and Wehr 2016) for the discretization schemes), we refer to Figure S1B to emphasize that diffusive lensing occurs not only under the Itô convention but across a wide parameter space. Indeed, it is absent only in the normative isothermal convention; note that even a stochastic differential equation conforming to the isothermal convention may be reformulated into the Itô convention by adding suitable drift terms, allowing for diffusive lensing to be seen even in case of the isothermal convention. We note in particular that the choice of the convention is a highly context-dependent one (Sokolov 2010); there is not a universally correct choice, and one can obtain stochastic differential equations consistent with Ito or Stratonovich interpretations in different regimes. Lastly, space-dependent diffusivity is now an experimentally well-

recognized feature of the cellular interior, as noted in our references and as discussed further later in this response. This fact points towards the potential relevance of our model for subcellular diffusion.

In our revised preprint, we have made changes to the text and minor changes to figures to address reviewer concerns.

Responses to the Reviewers

We thank the reviewers for their feedback and address the issues they raised in this rebuttal and in the revised manuscript. The central point that the reviewers raise concerns the validity of the drift-less Itô interpretation in modeling potential nonequilibrium types of subcellular transport arising from space-dependent diffusivity. If the drift term were considered, the resulting stochastic differential equation stochastic differential equation (SDE) is equivalent to one arising from the isothermal interpretation of heterogeneous diffusivity (Volpe and Wehr 2016), wherein no diffusive lensing is seen (as shown in Fig. S1B). That is, the isothermal interpretation and the drift-comprising Itô SDE produce the same uniform steady-state particle densities.

While we agree with the reviewers that for a given interpretation, equivalent stochastic differential equations (SDEs) arising from other interpretations may be drawn, we disagree with the generalization that all types of subcellular diffusion conform to the isothermal interpretation. That is, there is no reason why any and all instances of nonequilibrium subcellular particle diffusion must be modeled using isothermal-conforming SDEs (such as the drift-comprising Itô SDE, for instance). We refer to (Sokolov 2010) which prescribes choosing a convention in a context-dependent manner. In this regard, we disagree with the second reviewer's characterization of making such a choice merely a "choice of writing" considering that it is entirely dependent on the choice of microscopic parameters, as detailed in the discussion section of the manuscript. The following references have also been added to the manuscript: the reference from the first reviewer (Kupferman et al. 2004) proposes a prescription for choosing an appropriate convention based upon comparing the noise correlation time and the particle relaxation time. The reference notes that the Itô convention is appropriate when the particle relaxation time is large when compared to the noise correlation time and the Stratonovich convention is appropriate in the converse scenario. In (Rupprecht et al. 2018), active noise is considered and the resulting Fokker-Planck equation conforms to the Stratonovich convention when thermal noise was negligible. The related reference, (Vishen et al. 2019) compares three timescales: those of particle relaxation, noise correlation and viscoelastic relaxation, to make the choice. Indeed, as noted in the manuscript, lensing is seen in all but one interpretation (without drift additions); only its magnitude is altered by the interpretation/choice of the drift term. The appendix has been modified to include a subsection on the interchangeability of the conventions.

Separately, with regards to the discussion on anomalous diffusion, the section on mean squared displacement calculation has been amended to avoid confusing our model with canonical anomalous diffusion which considers the anomalous exponent; how the anomalous exponent varies with space-dependent diffusivity offers an interesting future area of study.

Responses to specific reviewer comments appear below.

> ***Reviewer #1 (Public Review):***
>
> *The manuscript "Diffusive lensing as a mechanism of intracellular transport and compartmentalization", explores the implications of heterogeneous viscosity on the diffusive dynamics of particles. The authors analyze three different scenarios:*
>
> *(i) diffusion under a gradient of viscosity,*

> *(ii) clustering of interacting particles in a viscosity gradient, and*
>
> *(iii) diffusive dynamics of non-interacting particles with circular patches of heterogeneous viscous medium.*
>
> *The implications of a heterogeneous environment on phase separation and reaction kinetics in cells are under-explored. This makes the general theme of this manuscript very relevant and interesting. However, the analysis in the manuscript is not rigorous, and the claims in the abstract are not supported by the analysis in the main text.*
>
> *Following are my main comments on the work presented in this manuscript:*
>
> *(a) The central theme of this work is that spatially varying viscosity leads to position-dependent diffusion constant. This, for an overdamped Langevin dynamics with Gaussian white noise, leads to the well-known issue of the interpretation of the noise term.*
>
> *The authors use the Ito interpretation of the noise term because their system is non-equilibrium.*
>
> *One of the main criticisms I have is on this central point. The issue of interpretation arises only when there are ill-posed stochastic dynamics that do not have the relevant timescales required to analyze the noise term properly. Hence, if the authors want to start with an ill-posed equation it should be mentioned at the start. At least the Langevin dynamics considered should be explicitly mentioned in the main text. Since this work claims to be relevant to biological systems, it is also of significance to highlight the motivation for using the ill-posed equation rather than a well-posed equation. The authors refer to the non-equilibrium nature of the dynamics but it is not mentioned what non-equilibrium dynamics to authors have in mind. To properly analyze an overdamped Langevin dynamics a clear source of integrated timescales must be provided. As an example, one can write the dynamics as Eq. (1) \dot x = f(x) + g(x) \eta , which is ill-defined if the noise \eta is delta correlated in time but well-defined when \eta is exponentially correlated in time. One can of course look at the limit in which the exponential correlation goes to a delta correlation which leads to Eq. (1) interpreted in Stratonovich convention. The choice to use the Ito convention for Eq. (1) in this case is not justified.*

We thank the reviewer for detailing their concerns with our model's assumptions. We have addressed them in the common rebuttal.

> *(b) Generally, the manuscript talks of viscosity gradient but the equations deal with diffusion which is a combination of viscosity, temperature, particle size, and particle-medium interaction. There is no clear motivation provided for focus on viscosity (cytoplasm as such is a complex fluid) instead of just saying position-dependent diffusion constant. Maybe authors should use viscosity only when talking of a context where the existence of a viscosity gradient is established either in a real experiment or in a thought experiment.*

The manuscript has been amended to use only "diffusivity" to avoid confusion.

> *(c) The section "Viscophoresis drives particle accumulation" seems to not have new results. Fig. 1 verifies the numerical code used to obtain the results in the later sections. If that is the case maybe this section can be moved to supplementary or at least it should be clearly stated that this is to establish the correctness of the simulation method. It would also be nice to comment a bit more on the choice of simulation methods with changing hopping sizes instead of, for example, numerically solving stochastic ODE.*

The main point of this section and of Fig. 1 is the diffusive lensing effect itself: the accumulation of particles in lower-diffusivity areas. To the best of our knowledge, diffusive lensing has not been reported elsewhere as a specific outcome of non-isothermal interpretations of diffusion, with potential relevance to nonequilibrium subcellular motilities. The simulation method has been fully described in the Methods section, and the code has also been shared (see Code Availability).

> *A minor comment, the statement "the physically appropriate convention to use depends upon microscopic parameters and timescale hierarchies not captured in a coarse-grained model of diffusion." is not true as is noted in the references that authors mention, a correct coarse-grained model provides a suitable convention (see also Phys. Rev. E, 70(3), 036120., Phys. Rev. E, 100(6), 062602.).*

This has been addressed in the common rebuttal.

> *(d) The section "Interaction-mediated clustering is affected by viscophoresis" makes an interesting statement about the positioning of clusters by a viscous gradient. As a theoretical calculation, the interplay between position-dependent diffusivity and phase separation is indeed interesting, but the problem needs more analysis than that offered in this manuscript. Just a plot showing clustering with and without a gradient of diffusion does not give enough insight into the interplay between density-dependent diffusion and position-dependent diffusion. A phase plot that somehow shows the relative contribution of the two effects would have been nice. Also, it should be emphasized in the main text that the inter-particle interaction is through a density-dependent diffusion constant and not a conservative coupling by an interaction potential.*

The density-dependence has been added from the Methods to the main text. The goal of the work is to present lensing as a natural outcome of the parameter choices we make and present its effects as they relate to clustering and commonly used biophysical methods to probe dynamics within cells. A dense sampling of the phase space and how it is altered as a function of diffusivity, and the subsequent interpretation, lie beyond the scope of the present work but offer exciting future directions of study.

> *(e) The section "In silico microrheology shows that viscophoresis manifests as anomalous diffusion" the authors show that the MSD with and without spatial heterogeneity is different. This is not a surprise - as the underlying equations are different the MSD should be different.*

The goal here is to compare and contrast the ways in which homogeneous and heterogeneous diffusion manifest in simulated microrheology measurements. We hope that an altered saturation MSD, as is observed in our simulations, provokes interest in considering lensing while modeling experimental data.

> *There are various analogies drawn in this section without any justification:*
>
> *(i) "the saturation MSD was higher than what was seen in the homogeneous diffusion scenario possibly due to particles robustly populating the bulk milieu followed by directed motion into the viscous zone (similar to that of a Brownian ratchet, (Peskin et al., 1993))."*

In case of i), the Brownian ratchet is invoked as a model to explain directed accumulation. We have removed this analogy to avoid confusion as it is not delved into further over the course of our work.

> *(ii) "Note that lensing may cause particle displacements to deviate from a Gaussian distribution, which could explain anomalous behaviors observed both in our simulations and in experiments in cells (Parry et al., 2014)." Since the full trajectory of the particles is available, it can be analyzed to check if this is indeed the case.*

This has been addressed in the common rebuttal.

> *(f) The final section "In silico FRAP in a heterogeneously viscous environment ... " studies the MSD of the particles in a medium with heterogeneous viscous patches which I find the most novel section of the work. As with the section on inter-particle interaction, this needs further analysis.*

We thank the reviewer for their appreciation. In presenting these three sections discussing the effects of diffusive lensing, we intend to broadly outline the scope of this phenomenon in influencing a range of behaviors. Exploring the directions further comprise promising future directions of study that lie beyond the scope of this manuscript.

> *To summarise, as this is a theory paper, just showing MSD or in silico FRAP data is not sufficient. Unlike experiments where one is trying to understand the systems, here one has full access to the dynamics either analytically or in simulation. So just stating that the MSD in heterogeneous and homogeneous environments are not the same is not sufficient. With further analysis, this work can be of theoretical interest. Finally, just as a matter of personal taste, I am not in favor of the analogy with optical lensing. I don't see the connection.*

We value the reviewer's interest in investigating the causes underlying the differences in the MSDs and agree that it represents a promising future area of study. The main point of this section of the manuscript was to make a connection to experimentally measurable quantities.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *The authors study through theory and simulations the diffusion of microscopic particles and aim to account for the effects of inhomogeneous viscosity and diffusion - in particular regarding the intracellular environment. They propose a mechanism, termed "Diffusive lensing", by which particles are attracted towards high-viscosity regions where they remain trapped. To obtain these results, the authors rely on agent-based simulations using custom rules performed with the Ito stochastic calculus convention, without spurious drift. They acknowledge the fact that this convention does not describe equilibrium systems, and that their results would not hold at equilibrium - and discard these facts by invoking the fact that cells are out-of-equilibrium. Finally, they show some applications of their findings, in particular enhanced clustering in the high-viscosity regions. The authors conclude that as inhomogeneous diffusion is ubiquitous in life, so must their mechanism be, and hence it must be important.*
>
> *Strengths:*
>
> *The article is well-written, and clearly intelligible, its hypotheses are stated relatively clearly and the models and mathematical derivations are compatible with these hypotheses.*

We thank the reviewer for their appreciation.

> *Weaknesses:*
>
> *The main problem of the paper is these hypotheses. Indeed, it all relies on the Ito interpretation of the stochastic integrals. Stochastic conventions are a notoriously tricky business, but they are both mathematically and physically well-understood and do not result in any "dilemma" [some citations in the article, such as (Lau and Lubensky) and (Volpe and Wehr), make an unambiguous resolution of these]. Conventions are not an intrinsic, fixed property of a system, but a choice of writing; however, whenever going from one to another, one must include a "spurious drift" that compensates for the effect of this change - a mathematical subtlety that is entirely omitted in the article: if the drift is zero in one convention, it will thus be non-zero in another in the presence of diffusive gradients. It is well established that for equilibrium systems obeying fluctuation-dissipation, the spurious drift vanishes in the anti-Ito stochastic convention (which is not "anticipatory", contrarily to claims in the article, are the "steps" are local and infinitesimal). This ensures that the diffusion gradients do not induce currents and probability gradients, and thus that the steady-state PDF is the Gibbs measure. This equilibrium case should be seen as the default: a thermal system NOT obeying this law should warrant a strong justification (for instance in the Volpe and Wehr review this can occur through memory effects in robotic dynamics, or through strong fluctuation-dissipation breakdown). In near-equilibrium thermal systems such as the intracellular medium (where, although out-of-equilibrium, temperature remains a relevant and mostly homogeneous quantity), deviations from this behavior must be physically justified and go to zero when going towards equilibrium.*

Considering that the physical phenomena underlying diffusion span a range of timescales (particle relaxation, noise, environmental correlation, et cetera), we disagree with the assertion that all types of subcellular diffusion processes can be modeled as occurring at thermal equilibrium: for example, one can easily imagine memory effects arising in the presence of an appropriate hierarchy of timescales. We have added references that describe in more detail the way in which the comparison of timescales can dictate the applicability of different conventions. We also refer the referee to the common rebuttal section of our response in which we discuss factors that govern the choice of the interpretation. The adiabatic elimination arguments highlighted in (Kupferman et al. 2004) provide a clear description of how relevant particle and environment-related timescales can inform the choice of stochastic calculus to use.

With regards to the use of the term “anticipatory” to refer to the isothermal interpretation, we refer to the comment in (Volpe and Wehr 2016) of the Itô interpretation “not looking into the future”. In any case, whether anticipatory or otherwise, the interpretation’s effect on our model remains unchanged, as highlighted in the section in the Appendix on the conversion between different conventions; this section has been added to minimize confusion about the effects of the choice of convention on lensing.

> *Here, drifts are arbitrarily set to zero in the Ito convention (the exact opposite of the equilibrium anti-Ito), which is the equilibrium equivalent to adding a force (with drift $-grad D$) exactly compensating the spurious drift. If we were to interpret this as a breakdown of detailed balance with inhomogeneous temperature, the "hot" region would be effectively at 4x higher temperature than the cold region (i.e. 1200K) in Fig 1A.*

Our work is based on existing observations of space-dependent diffusivity in cells (Garner et al., 2023; Huang et al., 2021; Parry et al., 2014; Śmigiel et al., 2022; Xiang et al., 2020). These papers support a definitive model for the existence of space-dependent diffusivity without invoking space-dependent temperature.

> *It is the effects of this arbitrary force (exactly compensating the Ito spurious drift) that are studied in the article. The fact that it results in probability gradients is trivial once formulated this way (and in no way is this new - many of the references, for instance, Volpe and Wehr, mention this).*

Addressed in the common rebuttal.

> *Enhanced clustering is also a trivial effect of this probability gradient (the local concentration is increased by this force field, so phase separation can occur). As a side note the "neighbor sensing" scheme to describe interactions is very peculiar and not physically motivated - it violates stochastic thermodynamics laws too, as the detailed balance is apparently not respected.*
>
> *The neighbor-sensing scheme used here is just one possible model of an effective attractive potential between particles. Other models that lead to density-dependent attraction between particles should also provide qualitatively similar results as ours; this offers an interesting prospect for future research.*
>
> *Finally, the "anomalous diffusion" discussion is at odds with what the literature on this subject considers anomalous (the exponent does not appear anomalous).*

This has been addressed in the common rebuttal, and the relevant part of the manuscript has been modified to avoid confusion.

> *The authors make no further justification of their choice of convention than the fact that cells are out-of-equilibrium, leaving the feeling that this is a detail. They make mentions of systems (eg glycogen, prebiotic environment) for which (near-)equilibrium physics should mostly prevail, and of fluctuation-dissipation ("Diffusivity varies inversely with viscosity", in the introduction). Yet the "phenomenon" they discuss is entirely reliant on an undiscussed mechanism by which these assumptions would be completely violated (the citations they make for this - Gnesotto '18 and Phillips '12 - are simply discussions of the fact that cells are out-of-equilibrium, not on any consequences on the convention).*
>
> *Finally, while inhomogeneous diffusion is ubiquitous, the strength of this effect in realistic conditions is not discussed (this would be a significant problem if the effect were real, which it isn't). Gravitational attraction is also an ubiquitous effect, but it is not important for intracellular compartmentalization.*

The manuscript text has been supplemented with additional references that detail the ways in which the comparison of timescales can dictate how one can apply different conventions. We refer the reviewer to the common rebuttal section of our response where we detail factors that dictate the choice of the convention to use. As previously noted, the adiabatic elimination arguments highlighted in (Kupferman et al., 2004) provide a prescription for how different timescales are to be considered in deciding the choice of stochastic calculus to use.

With regards to the strength of space-dependent diffusivity in subcellular milieu, various measurements of heterogeneous diffusivity have been made both across different model systems and via different modalities, as cited in our manuscript. (Garner et al. 2023) used single-particle tracking to determine over 100-fold variability in diffusivity within individual S. pombe cells. Single-molecule measurements in (Xiang et al. 2020) and (Śmigiel et al. 2022) reveal an order-of-magnitude variation in tracer diffusion in mammalian cells and multi-fold variation in E. coli cytoplasm respectively. Fluorescence correlation spectroscopy measurements in (Huang et al. 2022) have found a two-fold increase in short-range diffusion of protein-sized tracers in X. laevis extracts. We have also added a reference to a study that

uses 3D single particle tracking in the cytosol of a multinucleate fungus, A. gossypii, to identify regions of low-diffusivity near nuclei and hyphal tips (McLaughlinetal.2020). Many of these references deploy particle tracking and investigate how mesoscale-sized particles (i.e. tracers spanning biologically relevant size scales) are directly impacted by space-dependent diffusivity. Therefore, we base our model on not only space-dependent diffusivity being a well-recognized feature of the cellular interior, but also on these observations pertaining to mesoscale-sized particles' motion along relevant timescales.

These measurements are also relevant to the reviewer's question about the strength of the effect, which depends directly on the variability in diffusivity: for ten- or a hundred-fold diffusivity variations, the effect would be expected to be significant. In case of using the Itô convention directly, the contrast in concentration gradient is, in fact, that of the diffusivity gradient.

> *To conclude, the "diffusive lensing" effect presented here is not a deep physical discovery, but a well-known effect of sticking to the wrong stochastic convention.*

As detailed in the various responses above, we respectfully disagree with the notion that there exists a singular correct stochastic convention that is applicable for all cases of subcellular heterogeneous diffusion. Further, as detailed in (Volpe and Wehr 2016) and as detailed in the Appendix, it is possible to convert between conventions and that an isothermal-abiding stochastic differential equation may be suitably altered, by means of adding a drift term, to an Itô-abiding stochastic differential equation; therefore, one can observe diffusive lensing without discarding the isothermal convention if the latter were modified. Indeed, it is only the driftless (or canonical) isothermal convention that does not allow for diffusive lensing.

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