Wanted: A Positive Control for Anomalous Subdiffusion

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ABSTRACT Anomalous subdiffusion in cells and model systems is an active area of research. The main questions are whether diffusion is anomalous or normal, and if it is anomalous, its mechanism. The subject is controversial, especially the hypothesis that crowding causes anomalous subdiffusion. Anomalous subdiffusion measurements would be strengthened by an experimental standard, particularly one able to cross-calibrate the different types of measurements. Criteria for a calibration standard are proposed. First, diffusion must be anomalous over the length and timescales of the different measurements. The length-scale is fundamental; the time scale can be adjusted through the viscosity of the medium. Second, the standard must be theoretically well understood, with a known anomalous subdiffusion exponent, ideally readily tunable. Third, the standard must be simple, reproducible, and independently characterizable (by, for example, electron microscopy for nanostructures). Candidate experimental standards are evaluated, including obstructed lipid bilayers; aqueous systems obstructed by nanopillars; a continuum percolation system in which a prescribed fraction of randomly chosen obstacles in a regular array is ablated; single-file diffusion in pores; transient anomalous subdiffusion due to binding of particles in arrays such as transcription factors in randomized DNA arrays; and computer-generated physical trajectories.

INTRODUCTION

Much work is being done on anomalous subdiffusion in the plasma membrane, cytoplasm, and nucleus of cells, and in model systems. The main experimental questions: Is diffusion anomalous or normal, and what are the parameters describing it? The main theoretical question: What mechanism makes the diffusion anomalous? The main question linking these: How can the various mechanisms be distinguished experimentally?

Anomalous diffusion mechanisms and their identification are both highly active areas of research. A recent starting point in that literature is Magdziarz and Weron (1).

The area is controversial, especially the hypothesis that crowding causes anomalous subdiffusion. Höfling and Franosch (2) refer to "cellular crowding…identified by slow anomalous transport as its most distinctive fingerprint… ." Supporting this view are several sets of experiments on various model systems ((3–5); see also Hellmann et al. (6)). In the other view, Dix and Verkman (7) argue that "the notion of universally anomalous diffusion in cells as a consequence of molecular crowding is not correct…" and point out that subdiffusion may be an artifact of reversible photophysical processes, cell autofluorescence, or complexities in beam and cell geometry. Supporting this view are experiments on crowding models in which fluorescence correlation spectroscopy (FCS) results were explicitly found to be consistent with normal diffusion (8–10). The most direct comparison of methods was in recent NMR work by Shakhov et al. (11), who found normal diffusion in crowded dextran solutions like those in which Banks and Fradin (3) found anomalous subdiffusion by FCS. This NMR work has almost succeeded in making the NMR and FCS length scales overlap. Overlapping length-scales will make it possible to distinguish a crossover from an inconsistency between methods.

The experimental evidence on both sides has a major limitation. Those arguing against anomalous subdiffusion have no positive control, and those arguing for it have no calibration standard. In current practice, a control is done in a simple liquid to give normal diffusion, and then subdiffusion is or is not observed in the experimental system. A high priority for the entire field is devising a positive control for anomalous subdiffusion. In work on model crowding systems, differences in diffusion may be the result of differences in length scales, concentrations, tracers, crowders, or the relative sizes of tracers and crowders, or they may be the result of experimental artifacts. Having a common calibration would be advantageous in sorting out the other complexities. In work on cells, a physical calibration standard would reduce the need to use unfamiliar cell lines and proteins to resolve differences among laboratories.

This review emphasizes fluorescence measurements: FCS (12–14), fluorescence recovery after photobleaching (FRAP) (15,16), and single-particle tracking (SPT) (17–20). We assume the usual diffraction-limited length scales for these measurements. Pulsed-gradient spin-echo (PGSE), also known as pulsed field gradient (PFG), NMR measurements will not be discussed in detail here, but it will eventually be highly important to include them because they are an independent (orthogonal) measure of diffusion and they are

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potentially label-free. PGSE NMR measurements of anomalous diffusion are reviewed by Kärger and Stallmach (21).

ANOMALOUS SUBDIFFUSION

Anomalous subdiffusion is hindered diffusion in which the hindrances change the actual form of the time dependence, not just the numerical value of the diffusion coefficient. The mean-square displacement $\langle r^2 \rangle$ is

$$\langle r^2 \rangle \propto D_0 t \tag{1}$$

for normal diffusion,

$$\langle r^2 \rangle \propto t^{\alpha}, \alpha<1 \tag{2}$$

for pure anomalous subdiffusion, and

$$\langle r^2 \rangle \propto \begin{cases} t^{\alpha} & \text{for } t \ll t_{CR} \\ t & \text{for } t \gg t_{CR} \end{cases} \tag{3}$$

for transient anomalous subdiffusion. Here t is time, D_0 is the diffusion coefficient, α is the anomalous subdiffusion exponent, and t_{CR} is the crossover time. In other words, for anomalous subdiffusion the time dependence is nonlinear, specifically a power law; the diffusion coefficient is time dependent; and the conditions of the central limit theorem are not met (22).

Destainville et al. (23) brought up an important point about artifactual anomalous subdiffusion. To rephrase their comment (23) and my reply (24), there are two types of transient anomalous subdiffusion, transitional and true. In the transitional type, one mechanism operates at short times and another at long times. For example, in the Kusumi picket fence model of membrane corrals (25), there is fast short-range normal diffusion within the corral at a rate determined by lipid viscosity, and slow long-range normal diffusion determined by the corral size and the jump rate between corrals. As Destainville et al. (23) point out, there is necessarily a period of apparent anomalous subdiffusion between these two limits. A monotonic continuous curve of $\langle r^2 \rangle$ versus t with these limits necessarily has a region of slope < 1 in a log-log plot. But in the true type, varying some parameter increases the duration of anomalous subdiffusion, and in the appropriate limit yields pure anomalous subdiffusion. For example, for a random walk on an obstructed lattice, as the obstacle concentration increases to the percolation threshold, diffusion becomes more anomalous over longer times. At the percolation threshold, diffusion becomes anomalous at all times, with a known exponent.

In principle, transient anomalous subdiffusion is common, and is the result of obstructed diffusion with an obstacle concentration below the percolation threshold; binding models in which there is a deepest trap, and correspondingly, a longest escape time; and correlations that are long-time but not infinite. Unfortunately, few tools are available to detect it. It would be useful to have an experimental system with a tunable crossover in order to examine the effects of transient anomalous subdiffusion in FRAP, FCS, and PGSE NMR, and to verify its detectability in SPT.

Why does anomalous subdiffusion matter? First, it affects reaction kinetics through $D(t)$. If D is assumed to be constant but is in fact time dependent, the analysis of the time dependence of reactant concentrations is compromised. Second, anomalous subdiffusion is a probe of submicroscopic organization, though unfortunately far from uniquely invertible.

REQUIREMENTS

A calibration standard must meet a number of requirements:

Requirement 1

The standard must operate over the same length and timescales as the usual biophysical measurements. So diffusion must be anomalous over several μm and s for the optical measurements, shorter lengths for superresolution optical measurements (26,27), and longer lengths for fringe pattern FRAP and many PGSE NMR measurements. The length scale is fundamental; the timescale can be tuned via the viscosity of the medium.

If λ is the wavelength of light in the optical measurements, we need structures with feature sizes $\ll\lambda$. In percolation simulations, I have often used a 256 $\times$ 256 lattice with periodic boundary conditions. If the experimental lattice constant is taken to be 10 nm, the corresponding percolation cluster is 2.56 μm on a side, in the appropriate range for FRAP and FCS measurements. If the features are 5 nm diameter on 10 nm centers, the number density is $1 \times 10^{12}/\text{cm}^2$ to relate to pore densities and feature densities in the literature, or $6.35 \times 10^{12}/\text{in}^2$ to relate to the terabit per square inch densities in the semiconductor literature (terabit can be 10^{12} or 2^{40}). This pore size is within the mesoporous range as defined by the International Union of Pure and Applied Chemistry (IUPAC), 2–50 nm (28). For PGSE NMR, a high-field, high-gradient magnet gave a length-scale ~100 nm and was used to study the liquid-ordered/liquid-disordered phase separation in bilayers (29). A very specific aspect of the problem was pointed out by Cherdhirankorn et al. (30) for FCS in an inverse opal structure. If the void size is similar to the beam size, the result of any one measurement is highly sensitive to the exact position of the beam relative to the void.

A lower limit on size is that the structures ought to be macroscopic enough that the interaction of tracers and obstacles can be simply approximated, without atomistic modeling. The choice of feature size is thus a tradeoff. Small features give better averaging over feature structure in a diffusion measurement, but the interactions may become more complex to represent.

Requirement 2

The standard must be theoretically well understood, with a known anomalous subdiffusion exponent, ideally readily tunable. Well-understood mechanisms include percolation, the continuous-time random walk and the related finite hierarchy mechanism, single-file motion in a pore, synthesized motion, and fractional Brownian motion (though its microscopic mechanism is still an active area of research). The mechanism should not be a major research problem in its own right, such as reptation in a polymer melt.

In my opinion, crowding is an incompletely understood mechanism. Important experimental work has been done in several laboratories (3–5,11), but theory and modeling studies are still incomplete. One needs to know the exponent and the crossover length as a function of mobile obstacle concentration, size, and polydispersity, and the effect of immobile obstacles. Using crowding as a calibration standard is a circular argument, but crowding would still be useful for comparisons among techniques and laboratories, say using one anomalous system from Banks and Fradin (3) and one normal system (8–10). Diffusion of both tracer and crowder ought to be measured.

Requirement 3

The standard ought to be simple, reproducible, and noncryogenic. The standard ought to be independently characterizable—a stable nanostructure that can be characterized by some form of electron microscopy, or a statistically well-defined structure. If crowding becomes well enough understood to be used as a standard, the materials used ought to be commercially available and well defined in terms of composition, molecular weight, and polydispersity.

Requirement 4

If transient anomalous subdiffusion occurs, the anomalous regime ought to extend over two or three orders of magnitude, so the anomalous regime is distinct from transitional regions. Both the normal and anomalous regimes ought to be readily detectable. Ideally, the crossover would be tunable. Data should be analyzed in terms of a time-dependent exponent

$$\alpha(t) = \frac{d \ln\langle r^2\rangle}{d \ln t} \tag{4}$$

to show the width of transitions.

Requirement 5

For cross-calibration, the tracer must be detectable by both fluorescence and NMR. It would be advantageous to do the optical and NMR measurements on the same system but the differing concentration requirements make that difficult (31). FCS and SPT require low concentrations, FRAP requires moderate ones, and NMR requires high.

Requirement 6

The medium ought to be transparent and nonfluorescent. Insolubility would be advantageous, so that refractive index matching can be used to eliminate light scattering. An additional benefit is that index matching eliminates the van der Waals forces between the medium and the tracer (32).

Requirement 7

Spatial homogeneity is advantageous except in the case of anomalous subdiffusion due to a fractal substrate, where inhomogeneity on all length scales is of the essence. Multiple environments complicate interpretation of the results, as Sanabria et al. (33) pointed out in their work using silica sol-gels as obstructed diffusion models.

Requirement 8

The fluorophore should not enter long-lived dark states.

Comments

A key idea in this review is tunability—the ability to continuously vary some parameter that determines the anomalous diffusion exponent or the crossover time. As already mentioned, the tuning parameter for a random walk on an obstructed lattice is the obstacle concentration. A comprehensive example of tuning by obstacle concentration was given by Höfling et al. (34) for a three-dimensional continuum Lorentz model. In their simulations, a tracer moved by Newtonian dynamics among random overlapping obstacles, and underwent hard-sphere collisions with the obstacles. Motion was ballistic at short times, subdiffusive at intermediate times, and at long times diffusive or localized, depending on the obstacle concentration. These simulations yielded an entire family of curves of log $\langle r^2\rangle$ versus log t, showing clearly the various regimes and how they changed with obstacle concentration. We need similar results for anomalous subdiffusion due to crowding.

A standard idea in the literature is that the FCS curve for anomalous subdiffusion is difficult to distinguish from the curve for normal diffusion of a two-component mixture (35–37). These two cases can be distinguished by maximum entropy analysis (38,39). For a heterogeneous system, the maximum entropy algorithm yields the widest distribution of diffusion coefficients consistent with the FCS data. This algorithm was used to analyze experiments in terms of anomalous and two-component diffusion (3,33). A consistency check is in order, in which maximum entropy analysis is used to test the homogeneity of candidate calibration standards, and the calibration standard is used to test maximum entropy analysis. However circular this argument may sound, it is an improvement over experimental tests based only on normal diffusion in one- and two-component systems.

CROSS-CALIBRATION

A few articles have reported cross-calibration of optical and NMR measurements of normal diffusion, or between optical methods. Febo-Ayala et al. (40) compared PGSE NMR and FRAP in membranes. Grünwald et al. (41) showed that SPT and FCS diffusion coefficients for two proteins in aqueous solution agreed quantitatively. Gendron et al. (42) measured diffusion of various rhodamine labels in water by NMR to solve a key experimental problem in FCS—determination of the illuminated volume. This is commonly done using a known fluorophore as a calibration standard (12). In addition, Stasevich et al. (43) cross-calibrated FRAP and FCS to quantify photobleaching effects, and Adkins et al. (44) compared FRAP and FCS diffusion measurements in cells.

An important recent article by Feil et al. (31) compared NMR and SPT measurements of a rhodamine fluorophore in a nanoporous glass specially synthesized for this experiment. This work was undertaken as an experimental test of the ergodic hypothesis, and showed that the time-averaged D from SPT is the same as the ensemble-averaged D from pulsed field gradient NMR. As the authors discuss in detail, SPT requires low concentrations of tracer and pulsed field gradient NMR requires high concentrations, so the measurements could not be made at overlapping concentrations. But this work is as close a comparison as we have, with a concentration gap of only one order of magnitude.

CANDIDATE STANDARDS: ANOMALOUS

Length limitations do not allow detailed discussion in the text of candidate experimental standards for anomalous subdiffusion or hindered normal diffusion. Here we give an overview. In the Supporting Material, we review possible experimental systems in detail, bringing together work from the nanotechnology, biophysics, soft matter physics, and engineering literature. We discuss geometries, length-scales, and diffusion measurements, and provide keywords and starting points in the literature. Where possible, we use biophysical examples. The discussion here and the discussion in the Supporting Material follow the same outline. Extensive references are given in the Supporting Material. For anomalous subdiffusion, we discuss mostly the two-dimensional case for optical-scale systems. For obstructed normal diffusion, we consider the three-dimensional case.

NANOFABRICATION

The first type of candidate system is based on nanofabrication, which provides a wide variety of structures for obstructed diffusion. We consider arrays of nanodots, nanopores, and nanopillars to be equivalent here. In many cases, any one of these can be used as a template or mask to make another. At the level of this review, it is sufficient to identify an array with the appropriate feature size, spacing, and randomness. The emphasis is on the obstacles, not the required fluid phase.

For a tracer of nonzero radius, obstructed diffusion is controlled by the excluded area fraction, not just the area fraction of obstacles, so obstruction can be tuned by varying the size of the diffusing species. Near the percolation threshold, diffusion is highly sensitive to the size dependence of obstruction.

Regular lattice

Nanospheres on a flat substrate can self-assemble into a triangular lattice in which the ordering is driven by capillary forces during drying. The nanosphere array can be used as a mask for deposition of metal or etching of substrate. The resulting two-dimensional corral-like structures are expected to give free normal diffusion within the corrals and slow normal diffusion among the corrals, with anomalous subdiffusion over a limited length scale, tunable by varying the tracer size.

Regular arrays of parallel electrochemically etched pores can be generated. They may be useful as templates for nanopillars, or to allow FRAP experiments on a large number of single-file diffusion systems in parallel.

Perturbed lattice

To extend the length scale of anomalous subdiffusion, we consider perturbed lattice structures. One possibility is self-assembly of polydisperse nanospheres into irregular corrals. Another possibility is self-assembly of block copolymers. Diblock copolymers are a type of amphiphile consisting of two different immiscible polymer chains covalently linked end-to-end. The covalent linkage forces the separation of immiscible polymers to be on the length scale of the polymer chains. Some block copolymers form locally regular arrays of micelles that can be used to make a corresponding array of metal dots. Semiconductor fabrication is the main intended application, so the goal in the literature is to make a lattice of uniform metal dots homogeneous over the length scale of a silicon wafer. The conditions for regularity have been studied in detail. For the calibration problem, the regularity must be reduced.

Random nanostructures

We next consider random structures. An ideal random structure for calibration is a percolation cluster exactly at the threshold—a system studied extensively in the physics literature. Diffusion is anomalous at all length scales and the exponent is known.

Obstructed lipid bilayer

A highly biophysical approach is a supported bilayer with immobile obstacles, say gel-phase lipid domains,

immobilized transmembrane proteins, or nanofabricated structures that exclude the bilayer.

Randomly adsorbed nanospheres

So far, we have considered ordered and perturbed arrays of nanospheres, but another possibility is to use random deposition of nanospheres on a substrate sticky enough that the spheres cannot anneal to form an locally ordered structure.

Percolation by sputtering

One of the early experimental realizations of a percolating cluster was sputtered metal on a flat substrate. The electrical conductivity of the system was measured as a function of the amount of metal deposited, and the conductivity increased sharply at the percolation threshold.

Nanopillars

Extensive work has been done on so-called nanopillar forests of carbon fibers or carbon nanotubes, including very interesting measurements of diffusion in an aqueous phase obstructed by nanopillars.

Continuum percolation in an obstacle lattice

We propose a percolation problem of continuum percolation in an obstacle lattice, related to standard percolation but adapted to facilitate nanofabrication. Initially, a large regular lattice of obstacles is made by standard techniques of nanotechnology such as two-dimensional interference patterns in the extreme ultraviolet. Then randomly chosen obstacles are selectively ablated, for example by focused ion beam milling. Finally, diffusion is measured for a tracer large enough that it is trapped when it is in an intact patch of obstacles. As the fraction of ablated obstacles is increased, the range of diffusion increases, and eventually the system reaches the percolation threshold. The percolation threshold can be found by standard Monte Carlo methods, and anomalous subdiffusion can be characterized. An important experimental feature is that diffusion can be varied by varying the ratio of the tracer size to the obstacle lattice spacing. If the system is at the percolation threshold for tracers with diameter just below the lattice spacing, then smaller tracers will show transient anomalous subdiffusion and larger tracers will be trapped locally.

Pinholes

A thin film with random pinholes can be used as a mask to make obstacles.

Commercial membranes

Commercial membranes for filtration are manufactured by nuclear track etching or anodization of alumina. Unfortunately, the densities of pores are too low for the calibration problem, so custom-made membranes would be required. Similar work on nanowire arrays has been optimized for high density, and may be more applicable.

Three-dimensional fractals

Structures with three-dimensional fractal pores have been made by self-assembly of a three-dimensional random fractal, which is then used as a template to generate the porous solid.

Arbitrary patterns

The last sets of nanotechnology methods considered are the well-established ones able to make arbitrary patterns. These would have the great advantage that one could make nanostructures for which the anomalous diffusion properties are well understood from computer simulations, such as a percolation cluster. The disadvantage is that photolithography is limited by spatial resolution, and electron-beam lithography is limited by throughput.

BINDING

A second type of candidate system is based on binding to a finite hierarchy of binding sites in which weak binding sites are common and strong binding sites are rare. Diffusion is transiently anomalous, and adding layers of deeper traps to the hierarchy makes diffusion more anomalous for longer times. Biologically relevant arrays of binding sites such as DNA, protein, and aptamers can be made by standard methods to give the required distributions of concentrations and binding energies. One special requirement here is that the arrays must be random at the molecular level, not arranged as blocks of identical binding sites. The other special requirement is an initial state in which the tracer is out of thermal equilibrium with the binding sites. This nonequilibrium state could be produced by fast mixing of the tracer with the array of binding sites, or by photoactivation of the binding site on the tracer. If the tracer is equilibrated with the binding sites, diffusion is slow but normal at all times.

SINGLE-FILE DIFFUSION IN PORES

The third type is based on single-file diffusion in pores, which gives one-dimensional anomalous subdiffusion with a fixed exponent of 1/2. Diffusion is anomalous due to correlations. If the particles are required to move in single file, the motion of one particle requires the collective motion of many of its neighbors.

SYNTHETIC MOTION

The fourth type is synthetic motion, in which one generates the anomalous subdiffusion directly by mounting a stable

point fluorophore on a piezo stage and driving the stage using some anomalous subdiffusion algorithm. The advantages of this approach are that the different theoretical mechanisms for anomalous subdiffusion can be used, the actual response function of the entire optical system is tested, and the synthetic motion tests the effect of off-axis aberrations.

POLYMER SOLUTIONS

The final type of system uses polymers as a tracer, an obstacle, or both, specifically a labeled polymer chain in solvent, a fluorescent sphere in a polymer solution, or a labeled polymer chain in a polymer solution. Limitations are the short range of anomalous subdiffusion and the complexity of the dynamics.

CANDIDATE STANDARDS: NORMAL

It would also be useful to have standards for hindered three-dimensional normal diffusion. Strictly speaking, these are likely to have short periods of transitional anomalous subdiffusion, but the diffusion is to a good approximation normal.

Opals

Opals are made by self-assembly of nanospheres into a regular three-dimensional superlattice. Inverse opals are a superlattice of nanospherical voids made by filling the interstices of an opal with some material and then destroying the nanospheres. Both structures are, in the ideal case, regular three-dimensional corrals.

Mesoporous materials

Mesoporous materials are regular or irregular porous solids, often synthesized by polymerization in a system with a surfactant template. Regular mesoporous materials include some with arrays of parallel pores, potentially useful for FRAP measurements of single-file diffusion.

Phase-separated glasses

Phase-separated glasses are formed by spinodal decomposition of a glass, followed by leaching of one phase. Pores are irregular with branching, but do not have the sort of fractal structure that leads to anomalous subdiffusion in a percolation cluster near the threshold.

DISCUSSION

This review is intended to bring attention to the calibration problem, not to serve as territorial marking. I am planning to do simulations related to possible standards. For continuum percolation on an obstacle lattice, one needs to find the anomalous subdiffusion exponent, the crossover time to normal diffusion, and the long-range diffusion coefficient, all as a function of the excluded area. Similar calculations are needed for disordered lattices, as a function of the excluded area and the degree of disorder. I hope that experimentalists will try some of these approaches.

SUPPORTING MATERIAL

Sections S1 and S2, with one figure, eight equations, and references (45–251), are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(12)01192-7.

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