

Title:

The Shape of Intracellular Water: Questions, Tools, and Scales

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Abstract

Intracellular transport underlies virtually every biological process, yet the physical organization of the cytoplasm remains one of the central unresolved questions in cell biophysics. This chapter revisits the long-standing paradox between the nearly unhindered rotational diffusion of proteins in living cells and their substantially slower translational motion. Rather than following a linear path from question to answer, we recount how a long-standing biological paradox ultimately converged with the development of new approaches to spatiotemporal fluorescence fluctuation spectroscopy, culminating in image Mean Square Displacement (iMSD) analysis. By extending fluorescence correlation measurements into a previously inaccessible spatiotemporal regime, these methods revealed that inert proteins undergo unrestricted Brownian diffusion over distances up to approximately 100 nm before progressively encountering structural constraints imposed by the intracellular environment. This observation reconciled decades of apparently conflicting measurements and provided direct experimental evidence that molecular transport inside cells is intrinsically scale-dependent. We discuss how these findings led from the original interpretation of intracellular nanopools to the broader concept of structured porosity, and how subsequent advances in macromolecular crowding, biomolecular condensates and mesoscale cellular organization have reinforced the view of the cytoplasm as a hierarchically organized physical environment. More broadly, this chapter illustrates how advances in measurement reshape biological questions and, ultimately, how the architecture of the cell gives shape to the space accessible to water and molecules, reflecting a scientific perspective that has characterized Enrico Gratton's contribution to modern fluorescence biophysics.

Keywords: Intracellular diffusion; Spatiotemporal fluctuation spectroscopy; Image Mean Square Displacement (iMSD); Scale-dependent transport; Cytoplasmic organization; Macromolecular crowding.

1. The unresolved paradox

One protein, two physical worlds

The physical nature of the intracellular environment has challenged cell biologists and biophysicists for decades. Although the molecular composition of the cytoplasm is now known in remarkable detail, its physical organization remains much harder to define. Is the cytoplasm best described as a concentrated aqueous solution, a viscoelastic gel, a porous medium, or a dynamically reorganizing active material? Over the years, each of these descriptions has found convincing experimental support, suggesting that they capture different aspects of the same highly complex system rather than representing mutually exclusive models.

As illustrated in **Figure 1**, the problem ultimately crystallized into a remarkably simple paradox. The same inert protein, GFP, appeared to experience two fundamentally different physical worlds inside the cell. Rotational diffusion reported a local environment with water-like properties over nanometre distances, whereas translational diffusion was markedly hindered over larger spatial scales. Reconciling these apparently conflicting observations required direct experimental access to the intermediate spatiotemporal regime separating the two measurements—a challenge that ultimately motivated the work described in this chapter.

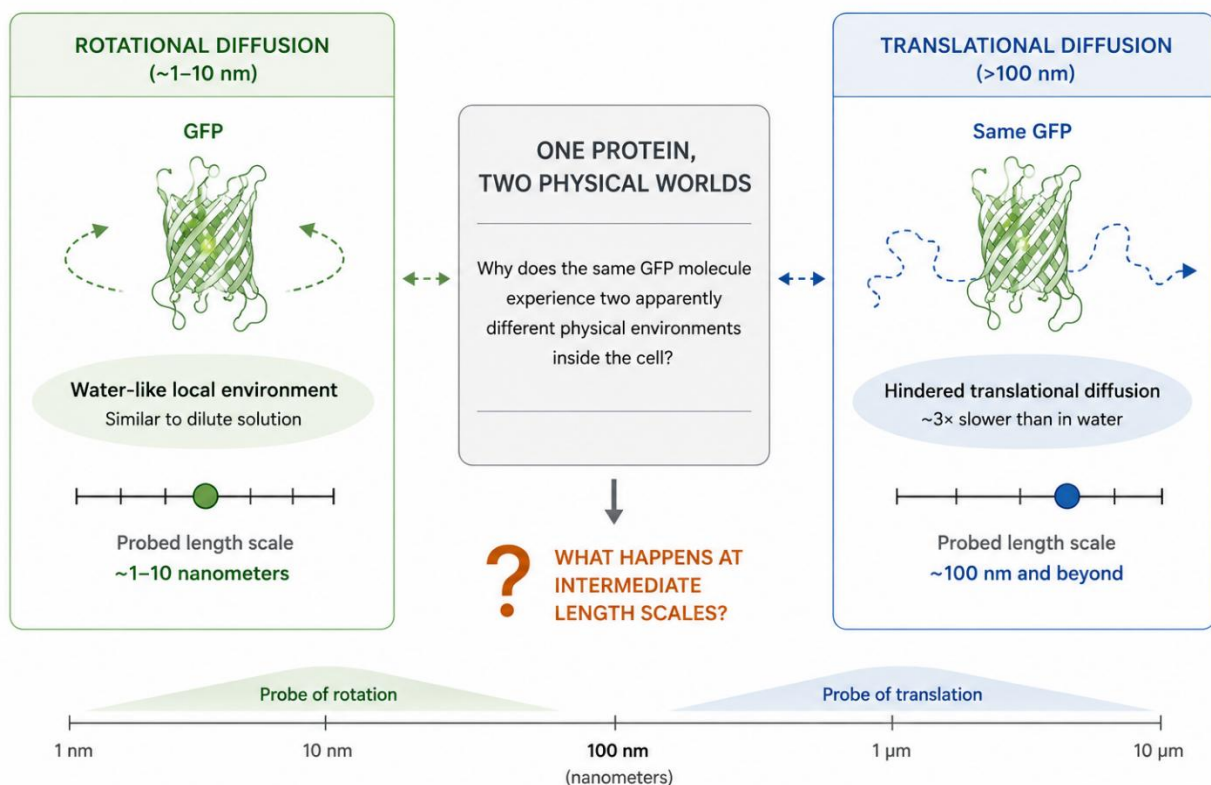


Figure 1 | One protein, two physical worlds: the unresolved paradox of intracellular transport.

Rotational and translational diffusion interrogate the intracellular environment over fundamentally different spatial and temporal scales. While GFP rotation reports a local environment with water-like properties, its translational motion is markedly hindered at larger distances. Reconciling these

apparently conflicting observations required direct experimental access to the intermediate spatiotemporal regime separating the two measurements—a challenge that motivated the development of the approaches described in this chapter.

Understanding this apparent contradiction is far from an academic exercise. Virtually every intracellular process depends on molecular transport. Enzyme kinetics, signal transduction, protein assembly and gene regulation all rely on the ability of molecules to explore the intracellular space. Diffusion inside living cells is therefore not merely a transport process; it is one of the fundamental physical determinants of cellular function. Understanding intracellular diffusion ultimately means understanding the physical organization of the cell itself. Beginning in the early 1990s, a series of elegant studies from Alan Verkman's laboratory progressively established what appeared to be an internally inconsistent physical picture of the cytoplasm. Early fluorescence anisotropy measurements showed that the local aqueous environment experienced by intracellular probes retained a viscosity close to that of water [1,2](Fushimi & Verkman, *Journal of Cell Biology*, 1991; Bicknese et al., *Journal of Biological Chemistry*, 1993). The introduction of green fluorescent protein (GFP) as an inert intracellular probe then made it possible to compare, within the same experimental system, both rotational and translational diffusion in living cells. Remarkably, while GFP rotational diffusion remained only marginally affected relative to dilute solution, its translational diffusion was found to be several-fold slower [3](Swaminathan et al., *Biophysical Journal*, 1997). Independent measurements of translational diffusion of macromolecular probes further confirmed that long-range transport in the cytoplasm was substantially hindered compared with dilute solution [4](Seksek et al., *Journal of Cell Biology*, 1997), consolidating the emerging view of the cytoplasm as a crowded intracellular environment [5,6](Luby-Phelps, *International Review of Cytology*, 2000; Verkman, *Trends in Biochemical Sciences*, 2002).

At first glance, these observations appeared irreconcilable. How could the same protein rotate almost as freely as it does in water while translating through an environment that behaved as if it were several times more viscous? Yet the apparent discrepancy did not go unnoticed by the authors themselves. In discussing their results, Swaminathan and colleagues proposed that rotational diffusion might remain largely unaffected because the spatial excursions associated with molecular rotation are much smaller than the characteristic spacing between intracellular obstacles. If the protein is smaller than the average distance separating these obstacles, rotational motion would remain essentially unrestricted, whereas translational motion over longer distances would progressively encounter steric barriers. Although qualitative, this interpretation was remarkably insightful. It implicitly introduced the idea that the physical properties experienced by a molecule inside the cell may depend on the spatial scale over which they are measured.

I vividly remember one of our weekly laboratory meetings in Irvine during my postdoctoral years in Enrico Gratton's laboratory. Rather than questioning the validity of Verkman's measurements, Enrico returned to this apparent paradox with a deceptively simple question:

"How can the same GFP molecule rotate as if it were in water, yet translate as if it were diffusing through a much more crowded environment?" What fascinated him was not the discrepancy itself, but the possibility that both observations were simultaneously correct. If so, the contradiction did not lie in the experiments, but in the conceptual framework used to interpret them. Looking back, that discussion anticipated a concept that has since become increasingly central to cell biophysics: the intracellular environment cannot be described by a single effective viscosity. Instead, the physical properties experienced by a diffusing molecule are intrinsically scale-dependent. Rotational diffusion probes the immediate hydration environment surrounding a molecule over nanometre distances and nanosecond timescales, whereas translational diffusion progressively samples increasingly larger portions of the intracellular space over microseconds, milliseconds and beyond. If the cytoplasm possesses a characteristic structural organization, then these apparently conflicting observations simply reflect different windows onto the same physical landscape.

At the time, however, this hypothesis remained experimentally inaccessible. Existing techniques could accurately measure either local rotational dynamics or long-range translational transport, but left unexplored the intermediate spatiotemporal regime where the transition between these two behaviours was expected to occur. The missing information lay precisely between these observational scales. Resolving this paradox therefore required observing molecular diffusion over spatial and temporal scales that had previously remained inaccessible to experiment.

2. A new way of observing molecular motion

From fluorescence fluctuations to spatiotemporal spectroscopy

Scientific advances are often presented as the direct consequence of a biological question. In reality, the opposite is frequently true. Entire classes of biological problems remain invisible until a new way of observing them becomes available. Looking back, this was one of the defining characteristics of Enrico Gratton's scientific approach. Rather than developing techniques to answer existing questions, he continuously sought new ways of extracting physical information from fluorescence measurements, convinced that every increase in observational capability would eventually reveal phenomena that had previously remained hidden. Fluorescence Correlation Spectroscopy (FCS) represented one of the milestones of this vision. Following its original conceptual introduction by Magde, Elson and Webb [7] and its subsequent methodological development [8], FCS demonstrated that spontaneous fluorescence fluctuations contain quantitative information on molecular concentration, diffusion and interactions at the single-molecule level. The introduction of one- and two-photon FCS further established fluorescence fluctuation spectroscopy as a powerful tool for investigating molecular dynamics directly in living cells [9]. By analysing fluctuations generated as molecules diffuse through a diffraction-limited observation volume, it became possible to quantify molecular transport with unprecedented temporal resolution. Yet,

despite its extraordinary sensitivity, conventional FCS remained intrinsically a local measurement. Molecular dynamics were inferred from fluorescence fluctuations occurring within a single observation volume, providing little information on the spatial organization of the environment through which diffusion occurred.

Enrico's key insight was that the microscope itself was collecting far more information than conventional analyses were extracting. During raster scanning, each pixel is acquired at a precisely defined instant after the previous one [10]. Images are therefore not purely spatial objects; they also contain an intrinsic temporal organization. Once this apparently simple observation is recognized, fluorescence images cease to be static representations of molecular distributions and become densely sampled spatiotemporal measurements of molecular motion. As schematically illustrated in **Figure 2**, the scan speed itself becomes a temporal filter, selectively probing progressively larger molecular displacements.

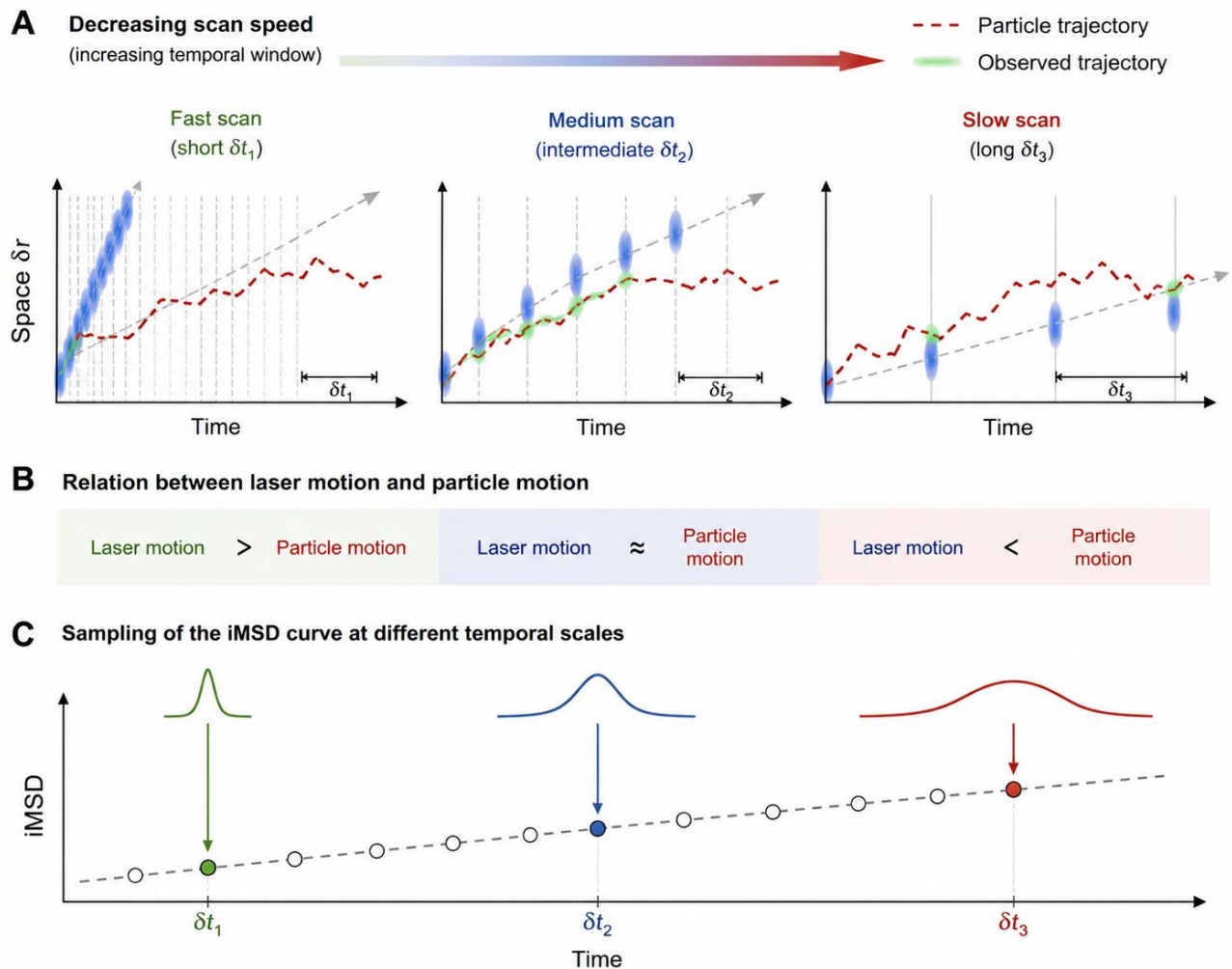


Figure 2 | Multiple scan-speed fluorescence fluctuation analysis enables reconstruction of molecular displacement across previously inaccessible spatiotemporal scales. Sequential raster scans acquired at progressively decreasing scan speeds sample increasingly larger molecular displacements over correspondingly longer temporal windows. When the laser scans much faster than molecular motion, the observed fluorescence profile closely reflects the microscope point spread function. As scan speed decreases, molecular motion progressively contributes to the broadening of the spatial correlation function. By extracting the width of the correlation peak at each

scan speed, the image Mean Square Displacement (iMSD) can be reconstructed as a function of the corresponding observation time. In this way, each scan speed acts as a selective temporal filter, allowing molecular motion to be sampled continuously across a broad range of spatial and temporal scales. This approach opens the previously inaccessible observational window between local rotational measurements and conventional long-range translational diffusion experiments. *Graphically redesigned and conceptually adapted from Di Rienzo et al., Nature Communications (Ref. [25]).*

This concept led naturally to the development of Raster Image Correlation Spectroscopy (RICS), in which the temporal structure hidden within raster scanning is exploited to recover molecular dynamics over an entire image rather than at a single observation point [11,12]. More importantly than the specific algorithm itself, RICS established a new conceptual framework: molecular transport could be investigated simultaneously in space and time. Imaging and spectroscopy were no longer separate experimental approaches but became different facets of the same measurement. This broader view of fluorescence fluctuation spectroscopy—as a quantitative framework for extracting physical information rather than simply measuring diffusion coefficients—was later articulated in Digman and Gratton's perspective on fluctuation correlation spectroscopy [13].

The same conceptual framework subsequently led to pair correlation analysis, which made it possible to identify barriers to molecular transport directly from fluorescence fluctuations without particle tracking [14]. The approach was subsequently extended to reveal molecular flow and transport pathways in living cells [15, 16], and has recently been consolidated into a comprehensive experimental framework for mapping intracellular transport by pair correlation microscopy [17]. Together, these developments progressively transformed fluorescence imaging from a descriptive imaging modality into a quantitative approach for probing the physical organization of living systems.

This evolving perspective was later synthesized in the concept of spatiotemporal fluctuation analysis, emphasizing how the integration of multiple imaging modalities and fluctuation-based approaches could provide a unified framework for investigating molecular transport and dynamic cellular organization beyond the diffraction limit [18].

Subsequent developments followed this same philosophy. Multiple scan-speed image correlation spectroscopy demonstrated that raster acquisition itself could become an experimental variable rather than a fixed instrumental parameter [19]. By varying pixel dwell time and line scanning speed, different portions of molecular trajectories become experimentally accessible. Scan speed therefore acts as a temporal filter, allowing progressively shorter or longer molecular displacements to contribute to the measured correlation function. Within this framework, we sought to move one step further. Instead of asking how fluorescence fluctuations decay over time, we asked whether they could reveal directly the evolution of molecular displacement itself. This question led to the development of the image Mean Square Displacement (iMSD) approach [20]. Building upon the mathematical formalism of Spatiotemporal Image Correlation Spectroscopy (STICS) [21],

which had demonstrated that the full space–time correlation function could be exploited to recover molecular displacement from image sequences, we extended this concept into the temporal regime of molecular diffusion. Rather than measuring velocity fields or slow protein fluxes, the temporal evolution of the spatial correlation function was analysed to reconstruct the mean square displacement of the molecular population directly from fluorescence images. Importantly, the method neither requires extraction of individual trajectories nor relies on an *a priori* diffusion model. Instead, the diffusion law emerges directly from the experimental data. This represented a fundamental conceptual shift. Classical fluctuation spectroscopy generally returns parameters such as diffusion coefficients or characteristic correlation times. By contrast, iMSD reconstructs the complete relationship between molecular displacement and observation time. Brownian motion, confinement, transient trapping or compartmentalization are not imposed through model selection but emerge directly from the shape of the experimental iMSD curve. In other words, the experiment no longer measures a diffusion coefficient; it measures how diffusion evolves across spatial and temporal scales.

Equally important was the experimental flexibility of the approach. Because the analysis relies on ensemble fluorescence fluctuations rather than on tracking isolated molecules, it remains applicable to densely labelled systems, dim fluorescent probes and genetically encoded fluorescent proteins—conditions under which conventional single-particle tracking rapidly becomes impractical. In this sense, iMSD naturally bridges two traditionally distinct experimental worlds: the statistical robustness of fluctuation spectroscopy and the physical interpretability of mean square displacement analysis.

At the time, however, we regarded iMSD primarily as a methodological advance—a new way to quantify molecular transport over broad spatial and temporal scales. We had not yet realized that it had inadvertently opened precisely the experimental window that had remained inaccessible in the long-standing diffusion paradox discussed in the previous section. Only later did we realize that the observational window opened by iMSD was precisely the one required to revisit that long-standing paradox. The methodological journey had unexpectedly brought us back to the biological question from which this story had begun.

3. Returning to the paradox

A hidden transport length scale in the cytoplasm

The methodological developments described in the previous chapter had been driven by the desire to extract more physical information from fluorescence fluctuations rather than by a specific biological problem. Yet, once the iMSD framework had become available, it became apparent that it offered precisely the experimental window needed to revisit the long-standing paradox of intracellular diffusion. For the first time, molecular displacements could be observed over the tens-of-nanometres and tens-of-microseconds regime separating rotational diffusion from conventional measurements of translational transport. The question

that had remained largely speculative since the pioneering studies of Verkman [3] could finally be addressed experimentally.

The central prediction was remarkably simple. If intracellular crowding resulted primarily from a homogeneous distribution of freely diffusing macromolecules, translational diffusion should already be hindered at the molecular scale [22, 23]. Conversely, if the dominant obstacles were spatially organized and relatively immobile, inert proteins should retain unrestricted Brownian motion over short distances, with diffusion becoming progressively hindered only after exploring larger portions of the intracellular environment [24]. These two classes of models made fundamentally different predictions, yet existing experiments lacked the spatial and temporal resolution required to discriminate between them.

To address this question, we adapted the iMSD framework to the three-dimensional interior of living cells by combining raster image correlation spectroscopy acquired at multiple scan speeds with direct reconstruction of the molecular mean square displacement [25]. Rather than using different pixel dwell times to calculate independent temporal autocorrelation functions, each scan speed was treated as a selective probe of molecular displacements occurring over a characteristic temporal window. In this way, the experiment reconstructed the average displacement of GFP continuously from sub-microsecond to millisecond timescales, corresponding to spatial scales from approximately 25 nm to several micrometres [25].

As an inert intracellular tracer, we selected monomeric GFP. The rationale was straightforward. GFP displays negligible specific interactions with the intracellular environment, its hydrodynamic properties are well characterized, and previous studies had already established it as a suitable reference molecule for comparing rotational and translational diffusion inside living cells [3, 26]. The first step was therefore to establish the Brownian reference. Measurements performed in dilute aqueous solution showed that the reconstructed iMSD followed the expected linear dependence over the entire temporal range explored, yielding a diffusion coefficient fully consistent with the known hydrodynamic radius of GFP [25]. The method therefore faithfully recovered classical Brownian diffusion under conditions in which no structural obstacles were present. The crucial question was what would happen once the same experiment was performed inside living cells. The answer is summarized in Figure 3.

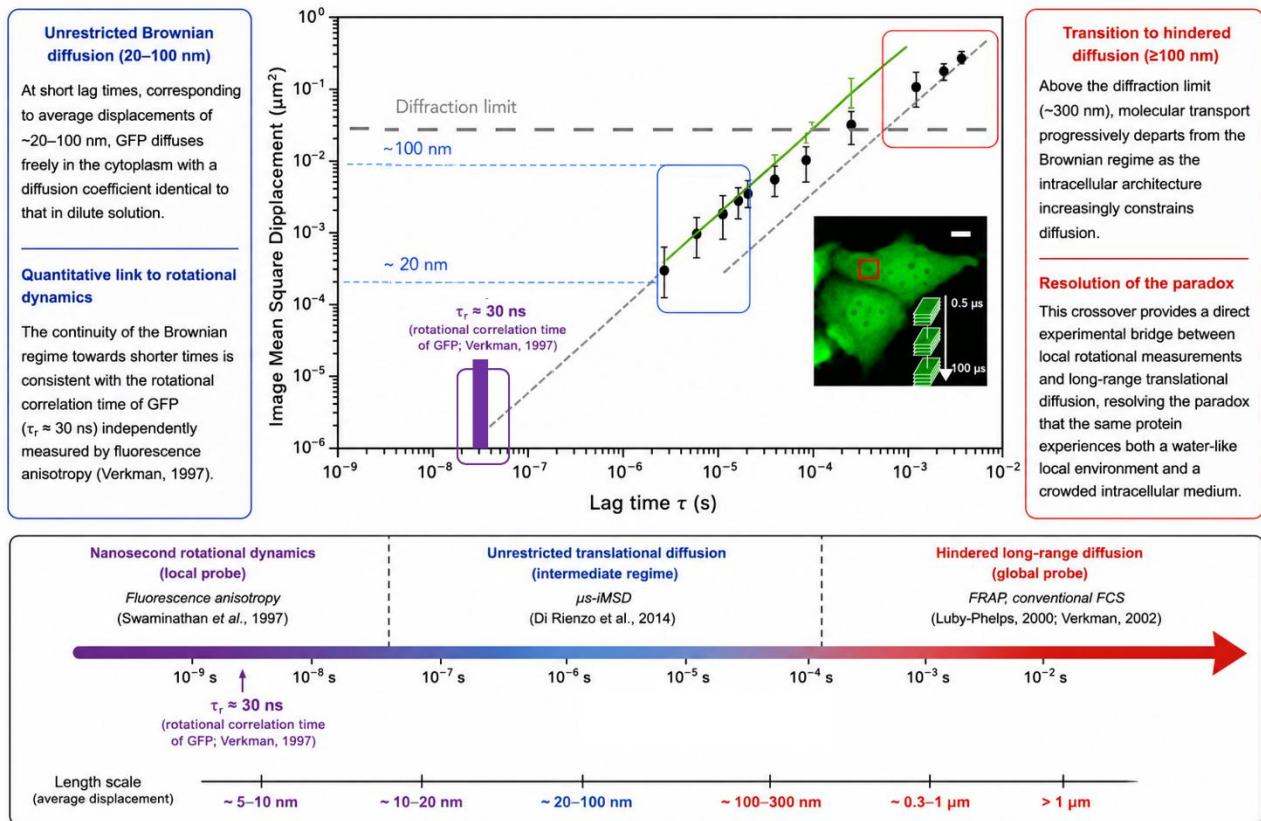


Figure 3 | Direct observation of a characteristic transport length scale in the cytoplasm. The image Mean Square Displacement (iMSD) of GFP in the cytoplasm reveals a scale-dependent transition in intracellular transport. At short lag times, corresponding to average molecular displacements of approximately 20–100 nm, GFP undergoes unrestricted Brownian diffusion, closely matching its behaviour in dilute aqueous solution. The continuity of this Brownian regime towards shorter times is quantitatively consistent with the rotational correlation time of GFP ($\tau_r \approx 30$ ns) independently measured by fluorescence anisotropy, suggesting that rotational and translational measurements probe different portions of the same underlying diffusion process across complementary spatiotemporal scales. Above the diffraction limit, molecular transport progressively departs from this Brownian regime as the intracellular architecture increasingly constrains diffusion. This crossover provides the missing experimental bridge between local rotational measurements and conventional long-range translational diffusion, resolving the long-standing paradox that the same protein appears to experience both a water-like local environment and a crowded intracellular medium. The lower schematic summarizes the corresponding spatiotemporal regimes explored by the different experimental approaches. *Graphically redesigned and conceptually adapted from Di Rienzo et al., Nature Communications (Ref. [25]).*

A strikingly different picture emerged when the same measurements were performed inside the cytoplasm of living CHO cells. At very short timescales—below approximately 2×10^{-5} s, corresponding to average molecular displacements between roughly 25 and 100 nm—the iMSD measured in the cytoplasm became indistinguishable from that measured in dilute solution [25]. Remarkably, the unrestricted Brownian regime identified by μ s-iMSD is fully consistent with the nanosecond timescale previously accessed by fluorescence anisotropy. Extrapolation of the experimental Brownian regime towards shorter times intersects the rotational correlation time of GFP ($\tau_r \approx 30$ ns) independently measured by Verkman and co-

workers [3]. Although these techniques probe different aspects of molecular motion—translation and rotation, respectively—the quantitative agreement is striking. It is unlikely to be coincidental. Rather, fluorescence anisotropy and μs -iMSD appear to sample different portions of the same underlying Brownian diffusion process, extending continuously from nanometre, nanosecond dynamics to molecular transport over hundreds of nanometres. Within this previously unexplored regime, GFP underwent essentially unrestricted Brownian diffusion, yielding an effective diffusion coefficient of approximately $126\ \mu\text{m}^2\ \text{s}^{-1}$ at $37\ ^\circ\text{C}$, virtually identical to that measured in water [25].

In retrospect, this observation provided the first direct experimental confirmation of the qualitative interpretation proposed years earlier by Swaminathan and colleagues [3]. Rotational diffusion had remained essentially unaffected because proteins explore only the immediate aqueous environment surrounding them. Translational diffusion, instead, progressively samples increasingly larger regions of the cytoplasm and eventually encounters structural barriers. What had remained hypothetical in 1997 now became directly observable: the cytoplasm possesses a characteristic transport length scale separating these two regimes of molecular motion.

The conclusion did not rely solely on observations in living cells. To discriminate between competing physical models of intracellular crowding, we reproduced the two limiting scenarios in simplified *in vitro* systems. In concentrated BSA solutions, representing a homogeneous population of freely diffusing crowders, GFP diffusion was uniformly reduced across all temporal scales. In contrast, nanostructured Sephacryl matrices reproduced the behaviour observed in living cells remarkably well: unrestricted Brownian diffusion at short distances followed by suppressed long-range diffusion as molecules encountered the structural boundaries of the porous network [25]. Together with numerical simulations and the absence of significant effects following microtubule depolymerization by nocodazole, these observations supported a model in which the dominant barriers to transport are relatively immobile spatial structures rather than a homogeneous population of freely diffusing soluble crowders [24, 25].

The most important outcome of this work was therefore not simply the observation of unrestricted Brownian diffusion. Rather, it was the experimental identification of a characteristic transport length scale in the cytoplasm. Once molecular motion was examined continuously across spatial scales, the apparent contradiction between rotational and translational diffusion disappeared. The quantitative continuity between rotational anisotropy and μs -iMSD further suggests that these techniques do not provide independent descriptions of intracellular transport. Rather, they probe different portions of the same underlying diffusion process across complementary spatiotemporal scales. The cell did not possess two different viscosities. Instead, it revealed a scale-dependent physical organization in which different transport regimes naturally emerge as progressively larger regions of the intracellular environment are explored.

4. The architecture of intracellular water

From nanopools to structured porosity

The experimental identification of a characteristic transport length scale naturally raises a broader question: what physical organization of the cytoplasm gives rise to this behaviour? The most important consequence of the observations described in the previous section is therefore not simply the identification of an unrestricted Brownian diffusion regime, but a different way of thinking about the intracellular environment itself. Our experiments did not directly visualize "nanopools" of intracellular water. Rather, they demonstrated that the cytoplasm behaves as if inert proteins experience locally water-like regions over short distances while progressively encountering structural barriers as increasingly larger portions of the intracellular space are explored. In this sense, the concept of nanopools should be regarded as a physical interpretation of a scale-dependent transport phenomenon rather than as the direct observation of discrete anatomical entities.

This interpretation naturally reconciles the long-standing discrepancy between rotational and translational diffusion. Rotational diffusion probes the immediate hydration environment surrounding a protein over nanometre distances, whereas translational diffusion progressively samples increasingly larger regions of intracellular space. Time-resolved fluorescence anisotropy had already shown that GFP rotation in cells is only weakly affected relative to dilute solution [1, 3]. Our iMSD measurements identified where this local water-like behaviour ceases: below approximately 100 nm GFP diffuses essentially as in dilute solution, whereas above this scale its motion becomes progressively constrained by the intracellular architecture [25].

Such behaviour is fully consistent with theoretical descriptions of diffusion in heterogeneous obstacle-laden environments. Lorentz-type and obstacle-based models predict that particles undergo normal Brownian diffusion over short displacements before progressively developing hindered or anomalous transport once their trajectories become comparable to the characteristic spacing between obstacles [27, 28]. The significance of our 2014 observations was that this crossover was not inferred indirectly from long-time diffusion measurements, but observed experimentally inside the three-dimensional cytoplasm of living cells.

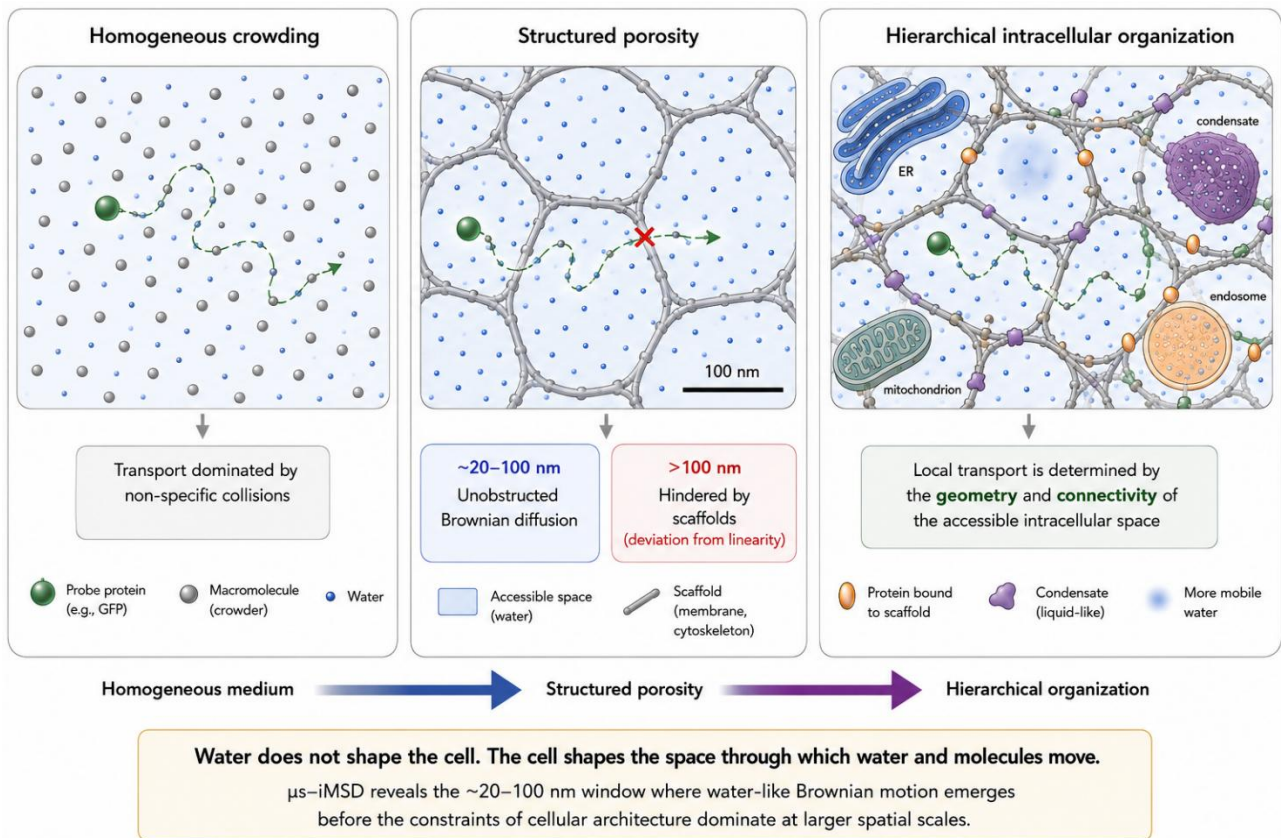


Figure 4 | From homogeneous crowding to hierarchical intracellular organization. The interpretation of intracellular transport has progressively evolved from a homogeneous view of macromolecular crowding to a scale-dependent picture in which molecular motion is governed by the geometry and connectivity of the accessible intracellular space. In the classical view, diffusion is slowed uniformly by collisions with freely diffusing macromolecules. The present work instead supports a model of structured porosity, in which proteins undergo unrestricted Brownian diffusion over short distances (approximately 20–100 nm) before progressively encountering relatively stable structural barriers. A decade of subsequent research has further expanded this picture, showing that the intracellular environment is organized across multiple spatial scales by membrane systems, organelles, cytoskeletal elements and biomolecular condensates, each contributing to the physical landscape explored by diffusing molecules. In this framework, molecular transport is determined not by a single effective intracellular viscosity, but by the hierarchical organization of the cellular architecture and by the topology of the space accessible to water and solutes.

With the benefit of the following decade, this interpretation can now be placed within a much broader conceptual evolution in cell biophysics. Following the classical view of the cytoplasm as a homogeneous crowded solution [29], the field has progressively embraced a picture of intracellular organization that is intrinsically heterogeneous across multiple spatial scales. Biomolecular condensates have established membrane-less compartmentalization as a fundamental organizing principle of cellular biochemistry [30]. At the same time, increasing attention has been devoted to distinguishing bona fide liquid-liquid phase separation from other forms of mesoscale molecular organization [31]. More recent work further emphasizes that condensates are dynamically regulated assemblies with material properties that continuously evolve rather than simple equilibrium liquid droplets [32]. Figure 4 summarizes

how these developments have progressively changed our physical interpretation of intracellular transport. Rather than replacing previous models, each step has expanded them, leading from the classical concept of homogeneous macromolecular crowding to a picture of structured porosity and, ultimately, to a hierarchically organized intracellular environment in which membranes, organelles, cytoskeletal structures and biomolecular condensates collectively define the physical landscape explored by diffusing molecules.

Importantly, these later developments do not directly demonstrate the existence of the nanopools proposed here, nor do they imply that biomolecular condensates explain the GFP diffusion crossover. Their significance is broader: they independently reinforce the concept that intracellular physical organization is intrinsically hierarchical, heterogeneous and dynamically regulated. Along the same lines, recent work has reframed molecular crowding itself as a physiological variable linked to cell volume, water mobility, macromolecular complex formation and phase separation rather than as a passive consequence of high macromolecular concentration [32].

Within this broader framework, structured porosity may represent a more general and physically meaningful description than the original concept of nanopools of water. The intracellular environment is neither a homogeneous viscous continuum nor a static gel. Instead, it behaves as a dynamically maintained architecture in which the geometry and connectivity of the accessible aqueous space govern molecular transport. Water may remain locally bulk-like over nanometric distances, but it is the topology of the surrounding cellular structures that gives shape to the space through which water and molecules move. This perspective also changes the meaning of intracellular viscosity. Rather than being treated as a single scalar property of the cytoplasm, viscosity emerges as a scale-dependent observable. At nanometric scales, inert proteins experience an effective viscosity close to that of water; at larger distances, the apparent increase in viscosity reflects the growing influence of cellular architecture on molecular trajectories rather than a change in the physical properties of water itself. Diffusion therefore becomes more than a transport process: it becomes a structural probe of the living cell. The characteristic crossover observed at approximately 100 nm is not simply a diffusion parameter, but a measurable physical signature of intracellular organization.

5. Questions, tools, and scales

Looking back at the story told in this chapter, what emerges most clearly is not a particular experimental technique or a single biological discovery, but a characteristic way of approaching scientific problems. The apparent contradiction between rotational and translational diffusion was never resolved by choosing one observation over the other. Instead, it was resolved by recognizing that the two measurements were probing different spatial and temporal scales of the same physical phenomenon.

This perspective was deeply embedded in the scientific culture of Enrico Gratton's laboratory. Discussions often began from experimental observations that appeared difficult to reconcile, not with the aim of identifying which result was correct, but with the assumption that every measurement contained genuine physical information. The challenge was therefore to understand what each experiment was actually observing and, equally importantly, what remained experimentally invisible. What remained with me throughout the development of this work was not a particular experiment or a single discussion, but a recurring way of approaching scientific questions. Apparent contradictions were rarely treated as problems to be resolved by choosing one observation over another. Instead, they were regarded as indications that the available measurements were probing different aspects of the same physical reality. The challenge was therefore not to decide which experiment was correct, but to understand what each experiment was actually measuring—and, equally importantly, what remained experimentally inaccessible.

This emphasis on observation rather than interpretation has characterized much of Enrico Gratton's scientific contribution to fluorescence spectroscopy. Throughout his career, methodological developments were rarely pursued as purely technical achievements. Instead, they were motivated by the conviction that extending the observational capabilities of an experiment would ultimately reveal new biological questions. In this sense, techniques such as fluorescence lifetime imaging, Raster Image Correlation Spectroscopy, pair correlation analysis and, more generally, spatiotemporal fluctuation spectroscopy were not simply new methods, but new ways of extracting physical information from living systems.

The work described in this chapter followed the same trajectory. The development of the iMSD approach was not originally conceived to solve the intracellular diffusion paradox. It emerged from a broader effort to recover the physical laws governing molecular motion directly from fluorescence images, without relying on particle tracking or predefined diffusion models. Only afterwards did it become evident that this new observational framework provided precisely the experimental window required to revisit a long-standing question in intracellular transport.

Looking at the field today, one may argue that the specific biological interpretation proposed in 2014 will continue to evolve, as every scientific model does. New experimental approaches, higher spatial resolution and increasingly sophisticated theoretical descriptions will undoubtedly refine our understanding of intracellular organization. What is less likely to change is the broader lesson illustrated by this story: progress often comes not from replacing existing observations with new ones, but from discovering how apparently conflicting observations become mutually consistent once they are viewed across the appropriate spatial and temporal scales. If there is one common thread linking the work described in this chapter with Enrico Gratton's broader scientific contribution, it is the conviction that advances in biology often follow advances in measurement. By extending the range of what can be observed, new experimental approaches do more than improve existing models—they redefine the questions that can be asked. Perhaps this is the most enduring lesson of the

story described here: before changing our models of nature, we should first ask whether we have learned to observe nature at the appropriate scale.

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