Biophysical Perspective

The Dark Matter of Biology

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ABSTRACT   The inside of the cell is full of important, yet invisible species of molecules and proteins that interact weakly but couple together to have huge and important effects in many biological processes. Such "dark matter" inside cells remains mostly hidden, because our tools were developed to investigate strongly interacting species and folded proteins. Example dark-matter species include intrinsically disordered proteins, posttranslational states, ion species, and rare, transient, and weak interactions undetectable by biochemical assays. The dark matter of biology is likely to have multiple, vital roles to regulate signaling, rates of reactions, water structure and viscosity, crowding, and other cellular activities. We need to create new tools to image, detect, and understand these dark-matter species if we are to truly understand fundamental physical principles of biology.

How many times have you read an article or performed an experiment on a reconstituted system that did not match with cellular data? This happens often in my field of cytoskeletal biophysics. Maybe the trends are similar, but the actual quantitative data are way off. One example is that the speeds of kinesin-1-driven cargos in cells are far faster than in vitro maximal speeds of the enzyme (1–3). How can that be? How can the motor speed up in the cell, especially when the cell interior is crowded and viscous and the motor is under load from other motors and the big cargos they transport? In fact, individual, truncated kinesin-1 motors are not individually faster in live cells (3). We can think of a million ways to slow the motor, yet it goes faster.

When such a discrepancy occurs, we give vague excuses. Our biochemistry and biophysics measurements are correct and repeatable, but the cell environment is weird and complex. There must be something we do not know. Something has been missed or overlooked, if only we could know the entire state of the cell. Many of us, biophysicists, have spent our careers trying to reconstitute biological processes in the microscope to visualize how they work, yet nothing we put together approaches the complexity that exists in the live cell. Further, even our best simulations and cartoons have an incomplete picture of the environment; we cannot recapitulate the statistical nature and feel of the cell interior.

All of this is confounded by the fact that we do not even know what is in the cell. A wonderful recently published book, Cell Biology by the Numbers by Milo and Phillips, does a great job of addressing the question of how many species of molecules and chemicals are in the cell (4). Measurements to determine the concentrations of different species can be performed on large numbers of cells and determined on average per cell. However, single-molecule biophysics has taught us that averages can be deceiving. Even if we made the measurement on a cell-by-cell basis, this still would be an average over the entire cell. Subcellular measurements of the local concentration, gradients, and dynamics of species can vary greatly and quickly. Further, many species exist at low copy numbers, making them virtually invisible to many cell-level or bulk measurements.

Given that we do not have fluorescent or dynamic reporters for most small molecules, how can we measure the local chemical state of the cell? The species that we cannot or do not detect are a part of the dark matter of biology. Like dark matter in gravitational physics, the dark matter of biology is interacting and performing functions that are perceptible, yet we cannot directly detect or sense the matter itself. Dark matter, in both physics and biology, is weakly coupled to the system, but we are only capable of detecting strongly interacting species. The result is a realization that the strong interactions we thought we understood are not correct in the context of the myriad interacting species of the cell. In this perspective review, I discuss some of the dark-matter species we need to explore. I describe where people are making inroads to begin to elucidate the activity of these species. Finally, I send out a challenge to the biophysics community to build new detection schemes to understand the dark matter of biology and focus more effort on weak and transient interactions.

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Dark-matter species

Ions

I start the discussion with the most mundane of dark-matter species: ions. The composition of salts inside of cells is highly regulated by membrane-embedded ion channels and pumps. Milo and Philips detail the average ionic nature inside and outside of cells (4), but what is the distribution of ionic species? Is there such a thing as an average cellular environment? For in vitro reconstitution, biochemists develop specialized buffers optimized for specific proteins and enzymes, but does the cell also have differential local buffers? How can we detect and quantify such local conditions?

There are a variety of metal ions that have specific functions in cells. Nearly 50% of proteins require the coordination of a metal ion species to function (4). The most interesting of these, working ions, are not the most abundant. For instance, calcium has many essential regulatory roles in the cell, but the levels are on the order of 10–100 nM, averaged over the entire cell (4,5). Calcium signaling is especially important in muscle, early development, and mitochondrial and endoplasmic reticular functions (5–7). Calcium regulates myosin motors and a number of actin-binding proteins that can regulate muscle contraction, such as troponin and tropomyosin (6,8). Yet calcium also causes rapid depolymerization of microtubules and inhibits many magnesium-requiring ATPase or GTPase enzymes that we know function in cells (9). This ability makes calcium an important regulator of bioprocesses that must be spatially regulated. Indeed, calcium ion density fluctuations have sharp gradients within the cell. These gradients are transitory, dynamic, and often oscillating. In some systems, they create beautiful patterns of traveling waves that can propagate and annihilate (7) (Fig. 1).

Other metal ions are equally important, and there are a number of specific proteins that bind, coordinate, or use
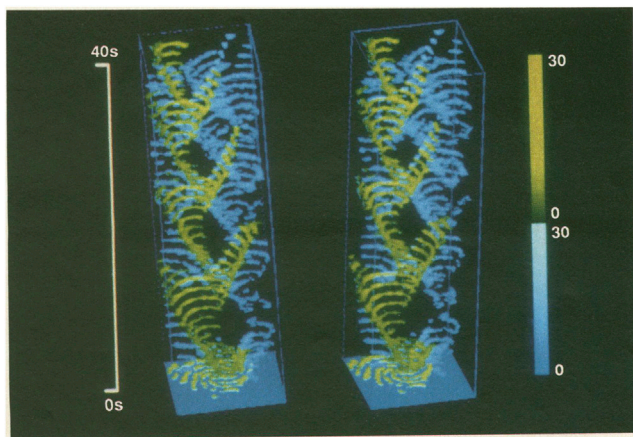

FIGURE 1 Calcium waves within *Xenopus* oocytes imaged using calcium-sensitive dyes. Image used with permission from *Science* magazine (7).

metal ions for specific functions. In particular, over half of all ATPase or GTPase enzymes require metal ions to function (10). Magnesium is obviously the most used, and it is highly abundant in cells (4). But cobalt, manganese, copper, nickel, and zinc ions are also extremely important (10). The abundance of these metal ions is low in cells, much like calcium, but they have large importance to many processes in cells of all types and are likely fluctuating, as calcium ions are.

Most ions are of relatively small valence, ± 1 or 2. Inorganic phosphate and iron species can carry up to a ± 3 charge. Other, polycationic species, such as polyamines of spermidine (+3) and spermine (+4) are also used for a number of processes in cells. In vitro studies show that high-valence species can cause "like-charge attraction" through electrostatic shielding (11–13). Spermidine especially is important to control membrane potential, intracellular pH, and volume. It has been shown to help longevity and diseases related to aging, stress, or protein aggregation (14,15).

Although we know they are important, we rarely know where metal ions are acting in live cells. Relatively new transition-metal-ion FRET sensors are not only sensitive sensors for structural FRET studies but can also be used to quantify the concentrations of metal ions in cells to track their locations and dynamics in time (16–19). Multiple sensors of ion species could be used to determine whether certain ions coordinate with, interact with, or ignore each other. Such sensors are important in continuing to shine light on ionic dark-matter species.

Viscosity and crowding

The interior of the cell is a "complex fluid," which is defined as a mixture with "the presence of a mesoscopic length scale which necessarily plays a key role in determining the properties of the system" (20). The cell interior has agents at a variety of length scales from small molecules to macromolecules, to complexes, to organelles. The incorporation of all these multiscale components results in the mesoscopic-length-scale interactions required for a complex fluid. Katherine Luby-Phelps has written several nice articles reviewing the literature while giving a wonderful description and intuition about the cell interior (21,22), so I will not rehash it here. Echoing the concepts of the complex fluid, Luby-Phelps concludes that the interior of the cell is a heterogeneous, compartmentalized volume.

Measurements of inert molecules diffusing in the cytoplasm of cells have been performed for the past 30 years (21). Researchers making such measurements have carefully created inert, noninteracting particles, and they often report a reduced mobility. Interestingly, the diffusion does not scale linearly with the size of the probes, as one would expect from models of diffusion in bulk liquid (21,23). Recent reviews on quantitative measurements of diffusion have focused on the anomalous nature of the mobility that

does not scale linearly with time as models of diffusion would dictate (22,24,25).

Much of this anomalous diffusion may be due to interactions of biomolecules throughout the cell (22). Although measuring the background solvent is important, it may have little effect on the mobility of interacting biomolecules, which can slow down or speed up depending on their activity and function. Oddly, small proteins are often faster in cells than inert molecules (26), likely due to their interactions with other species within the cell. The mobility (not necessarily diffusion) of biomolecules probably reports more on their interactions and activities and less on their size and ability to diffuse in open water.

Although she does not specifically say it, Luby-Phelps's writings imply that the cell can tune or alter both the viscosity and crowding within the cell at a local level. Given that eukaryotic cells control so many aspects of their structure and interior, we would be short-sighted to assume that they cannot or do not control their crowding and viscosity, as well. In previous works, inert mobility sensors that can detect both translational and rotational diffusion have been measured using fluorescence correlation spectroscopy with polarization detection (27–29). These reports have been extensively reviewed and modeled (23,25). Continued measurements of local diffusivity, rotational diffusion, and membrane diffusion of interacting species are needed to inform on the mobility and dynamics of interacting dark-matter species in cells.

Intrinsically disordered proteins and domains

When most biophysicists think about proteins, we envision compact, folded structures of α-helices and β-sheets. We discuss enzymes that act as tiny nanomachines performing work at the expense of ATP molecules. This picture of protein structure and function has been built on our technical ability to visualize protein structures using crystallized proteins. Unfortunately, we are unable to visualize intrinsically disordered or floppy domains of proteins using this technique, because such domains and structures do not align regularly in crystallized proteins. Thus, they are considered part of the dark matter of biology.

How common are intrinsically disordered domains? Almost 40% of proteins identified in the genomics data have a stretch of 50 or more amino acids that is intrinsically disordered or unstructured (30–32). Relaxing the length of the disordered region to only 10 amino acids causes the percentage to jump to 70% of all proteins (Fig. 2). A significant fraction of proteins, 15%, are completely disordered, with no discernible secondary structure. Many of these intrinsically disordered proteins are important and relevant to disease states (32–34).

In my field of the microtubule cytoskeleton, there are obvious examples of intrinsically disordered proteins. For instance, tau protein, which is important in axon develop-
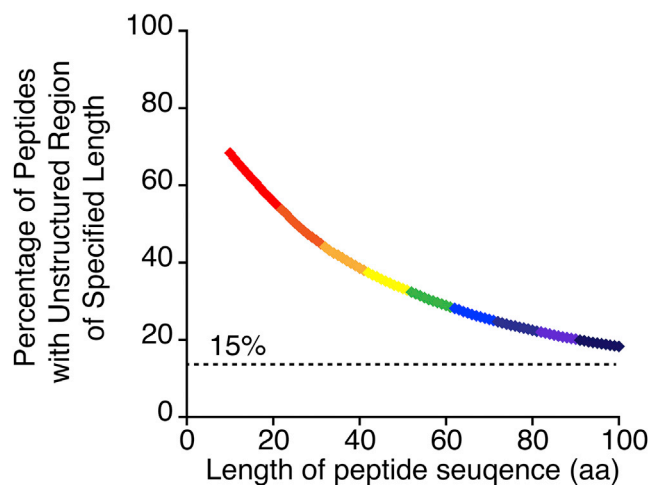

FIGURE 2 Percentage of protein sequences with at least one intrinsically disordered region of specified length plotted as a function of the specified length in amino acids. To see this figure in color, go online.

ment and maintenance (35), appears to be a completely disordered protein by many measures (36–38). Ordering and structure are actually bad things for tau and cause it to aggregate into neurofibrillary tangles that are associated with frontotemporal dementia and Alzheimer's disease (35,39).

Even the tubulin dimer, which has a highly conserved folded structure from yeast to humans, has a short, yet critically important, intrinsically disordered region: the carboxy-terminal tail. The carboxy-terminal tails are not only disordered but are highly charged, consisting of many glutamic acid residues, highly posttranslationally modified, and short (10–18 amino acids) (40). It has been known for decades that the carboxy-terminal tails of tubulin are essential to microtubule form and function (41). Indeed, you cannot even label this most obvious position with a GFP without destroying tubulin's ability to polymerize properly in cells (41).

Once intrinsically disordered proteins and regions were detected, the initially theorized mechanisms for their activities and functions were still biased by our macroscopic world views and technological inability to visualize working machines as anything other than compact objects. Thus, initial mechanistic models for intrinsically disordered polypeptides assumed that they took on folded conformations upon binding so that they could do productive work. Much research has focused on trying to observe such induced or transient folded states (42), but many attempts have failed. Perhaps these states do not exist and are merely a product of wishful thinking based on our preconceived notions of protein structure-function relationships and our inability to conceptualize floppy bits of string doing productive work.

Disordered proteins likely act as polymers, taking on many conformations that are determined by statistical

mechanics considerations. When free in solution, there are a countably infinite number of specific conformational states that the polypeptide chain can adopt, which can be characterized as "random coil" states. As the solution becomes crowded, random coils have been shown to elongate and extend (43).

Upon encountering a binding partner, the charged polypeptide would confront a new potential landscape determined by electrostatic and hydrophobic intramolecular forces. Binding within this new potential can enhance or decrease particular conformations of the random polypeptide, but there could still be a multitude of favorable "states." Peter Tompa has written several reviews and a book about intrinsically disordered proteins in which he describes the statistical nature of these types of proteins (32,42). He terms the multitude of states "fuzzy states." Such fluctuating conformational states could still have large, important effects but not rely on the classical "structure" at all. Further, the statistical lessons learned from disordered proteins should probably be applied to "properly folded" proteins and enzymes, which are also likely to sample a variety of states as they fluctuate among conformations, despite the static pictures returned by crystallography.

Just because they do not have a defined "structure" does not mean that intrinsically disordered proteins and domains cannot do work or create forces. Disordered domains can act as entropic springs and molecular spacers, purposely regulating the distance between structured colloidal regions or objects. If this is a main function of intrinsically disordered domains, then the length and mechanical properties of the disordered region are important factors. Specifically, these physical properties will tune the probability of two regions or proteins coming into contact.

If intrinsically disordered proteins can act as spacers, the purpose of tau protein binding to axonal microtubules may be to deliberately space apart microtubules (44). Much previous work has focused on the ability of tau to inhibit motor transport by acting as a roadblock (45–48), but if the microtubules are not spaced apart, there will be fewer tracks for motors to access. In a crowded environment, microtubules are more likely to condense due to depletion forces arising from osmotic pressure due to crowding. Tightly bundled microtubules will block the binding sites on neighboring filaments (49,50). Lack of access to the microtubule surface will impede transport, so the spacing by tau may be a necessary condition to enable transport.

We have shown that kinesin-1 single-molecule transport along microtubule bundles was aided by the presence of a transient microtubule cross-linker (MAP65) that spaced apart the microtubules (49). Recent studies with tau have shown that it is also a transient binder to the microtubule (48,51). Thus, although it was reported to be a roadblock, tau could enable, rather than inhibit, transport when considering crowded systems, such as in the cell. The question remains whether this is a consistent mechanism to control spacing and activity in cellular systems. Can large, floppy, transiently binding species help clear space and make room in an overly crowded cell interior to facilitate transport?

Intrinsically disordered domains can also act as sensors. Specifically, if an intrinsically disordered domain is free to bunch into a random coil, it could inhibit or hide binding sites. Upon extension, the random coil domain can open to reveal such cryptic binding sites. This could enable mechanosensation and mechanotransduction required in many cellular processes, including cell motility, cell division, and muscle contraction. Indeed, many intrinsically disordered proteins are known to operate within macromolecular complexes used in these processes. It is still being explored whether the "spring-like" or the "sensor-like" properties are more important mechanisms for controlling and sensing in these systems.

It is clear now that intrinsically disordered proteins and domains are immensely important, and many more biophysicists are starting to focus on this previously understudied dark-matter species. A topical subgroup at the Biophysical Society Meeting on Intrinsically Disordered Proteins started in 2007. In 2016, talks ranged from discussions of neurodegenerative diseases to phase separation in transient membraneless organelles. Experimental techniques included direct imaging with microscopy, NMR, and fast AFM. Theoretical techniques included molecular dynamics simulations to statistical mechanics calculations. There is also a website for mapping interactions of intrinsically disordered regions, called the Human Dark Proteome Initiative (https://darkproteome.wordpress.com). Given that most proteins at least have intrinsically disordered domains of importance, the new information learned by these groups will likely inform all of our work, shedding light on this dark-matter species.

Posttranslational modifications

The idea that posttranslational modifications (PTMs) of proteins are important and need to be examined is, perhaps, obvious. Anyone who has looked at any process involving "signaling" knows that phosphorylation, acetylation, glutamylation, and a variety of other modifications are used to communicate and talk throughout the cell (intracellular signaling) and between cells (extracellular signaling) (52).

Interestingly, many intrinsically disordered proteins and domains exhibit a multiplicity of posttranslationally modified states, adding further complexity to the discussion of the statistical nature, binding sites, and mechanics of such protein states (34). For an example from the cytoskeleton world, the intrinsically disordered carboxy-terminal tail of tubulin is highly modifiable in a variety of ways by processes including detyrosination, polyglutamylation, and polyglycosylation (53–55). These PTMs occur on filaments—not tubulin dimers—and are known to alter the

binding and activity of motor proteins, enzymes, and stabilizers (54,55). The downstream effects can alter network formation, rigidity, stability, and cargo transport.

Cells are like tiny computers. They receive inputs, they perform calculations, and they respond. The calculations they perform are done within signaling networks written in the language of PTMs of proteins. These proteins have already been translated and folded (to the extent that they are folded) using a more basic genetic code written in the DNA of the organism.

Using the computer analogy, the genetic code, written in DNA, acts like the operating system of the cell. The operating system is important for directing the underlying activities for creation of hardware (proteins), but it does not inform us about how the proteins talk to each other and interact. The signaling code, written in PTMs, is like a software program, also called applications, or "apps" for short. Previous reviews have used the description of a code to envision the posttranslational states of tubulin's carboxy-terminal tail (54–56). However, the code for the apps is probably not a simple one-to-one cipher. Rather, the PTM code is likely combinatorial, statistical, and complex in nature. Further, different codes can be written, erased, and rewritten on the same proteins repeatedly, adding complexity over time and space.

Unfortunately, we are currently at a technological impasse in trying to read the posttranslational code of the cellular apps. We cannot dynamically read or understand the posttranslational state of proteins, let alone monitor their dynamics or localization with time. New tools need to be created to read the posttranslational state of signaling systems both spatially and temporally in cells. Current methods include antibody staining and expressing proteins in mimicked states by replacing amino acids with others that have properties similar to the modified state. Antibody staining will reveal local states at specific times, but it is not dynamic or possible in live cells. Protein expression can reveal downstream effects of the signal, but it only works to misregulate the signals. Such methods give us the limiting cases of fully "on" or fully "off" but none of the nuance of true regulation. As such, current methods are incapable of elucidating the spatial or temporal changes as the code is read or written. All current observation methods lead to collapse of the system into an unnatural, nondynamic state. Truly new measurement techniques are needed to read out the posttranslational state of a population of proteins without adversely affecting the process being monitored— no small challenge.
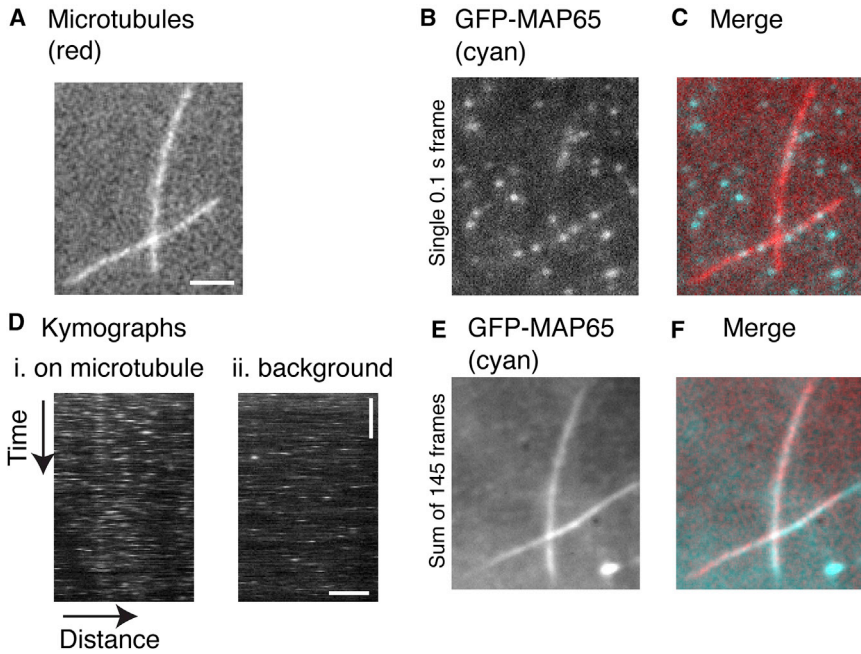
Weak interactions

Many of the above examples have highlighted the role of transient and weak interactions that appear to have large effects when multiplied. Weak and transient interactions are easy to miss or ignore because they are difficult to detect. Instead, most initial studies focused on strongly interacting species that have long binding times. This makes sense, because weak or transient interactions would be difficult to determine in dilute biochemical assays such as sedimentation or affinity pull-down assays. Further, the method of detection, often electrophoresis of proteins, has limited sensitivity—even when enhanced with antibody staining.

In my own research, we have examined the roles of weakly or even nonspecific binding on long-distance transport and cytoskeletal organization. For instance, we have shown that nonspecific interactions of kinesin-1 with cargos can greatly enhance the run length and association times of cargos even when single motors are unable to stay bound (57). We have further shown that weak, transient microtubule-cross-linking proteins can overpower strong-binding kinesin-1 motors to dictate networks of microtubules (58). Finally, we have shown that Eg5, the tetrameric kinesin motor responsible for spindle organization, is a fast, transient binder in live cells (59).

Weak, transient interactions are almost impossible to detect with traditional biochemical assays. This is because conventional biochemical experiments rely on strong interactions that can withstand long preparation times and dilute conditions. More modern, dynamic, sensitive fluorescence biochemical techniques can detect weak interactions in a population but can be difficult to interpret. We, and others, have used direct microscopy imaging with single-molecule sensitivity to visualize weak, transient fluorescent species as they interact. Such fast interactions can be visualized by a number of methods, such as kymography and collapsing time series by averaging over time. As an example, we detected transient interactions of MAP65, an antiparallel cross-linking microtubule-associated protein, using total internal reflection fluorescence microscopy (Fig. 3) (58). Interestingly, individual MAP65 molecules appear to not bind single microtubules at all in a single frame (Fig. 3, *B* and *C*). When the movie is collapsed over time, there is clearly enhanced binding to the microtubule, despite individual binding events spanning <100 ms (Fig. 3, *E* and *F*). We also performed similar analysis on Eg5 in live cells, which transiently and weakly interact with antiparallel microtubules, despite having a well-documented essential role in spindle elongation during cell division (59). Techniques of direct imaging via time-lapse microscopy and postanalysis can reveal such transient interactions. If performed quantitatively, it may be possible to extract weak equilibrium dissociation constants, although no study has yet shown this is possible. It is certainly a technique that, once developed and verified, could lead to measurements of weak interactions in a variety of systems both in vitro and in live cells.

We predict that weak interactions are not only common, but immensely important in the cell. Such weak interactions can locally increase the concentration of important

A Microtubules
(red)

B GFP-MAP65
(cyan)

Single 0.1 s frame

C Merge

D Kymographs

i. on microtubule   ii. background

Time

Distance

E GFP-MAP65
(cyan)

Sum of 145 frames

F Merge

FIGURE 3 Transient interactions of MAP65 binding to microtubules. (*A*) Epifluorescence image of microtubules. (*B*) Individual 100 ms frame of single molecules of GFP-MAP65 binding to microtubules. (*C*) Merged image of the microtubule (*red*) and GFP-MAP65 (*cyan*) to show that some molecules are bound on the microtubules and others are randomly in the background. (*D*) Kymograph of GFP-MAP65 binding to microtubules (*i*) or in the background in a region not on microtubules (*ii*). It is clear that MAP65 interacts more often with the region on the microtubule, but each interaction is brief, one to two frames at 100 ms/frame. (*E*) Collapsing the movie of GFP-MAP65 by summing the frames confirms the kymograph results that the GFP-MAP65 is binding more frequently to the microtubules than to the background. (*F*) Merged overlay of microtubules (*red*) on collapsed movie (*cyan*) shows an exact correspondence between the GFP-MAP65 binding and the microtubule location. Data are from (58).

molecules in a specific region. Indeed, signaling cascades are most likely the culmination of many weakly interacting molecules rather than a few strongly interacting ones. Such activities can lead to altered transport and diffusion, local sorting, and gradients of molecules and proteins, as well as the formation of larger complexes and phase separations. Indeed, there has been a recent resurgence of the idea of liquid-liquid phase separation of proteins to create membraneless organelles. This idea dates back to a publication by E. B. Wilson in 1899 (22,60,61). Currently, high-resolution fluorescence imaging techniques in live cells offer direct visualization of liquid-like phases of proteins as they transiently form and dissolve. By altering the expression, and thus the concentration of the constituent proteins in situ, biophysicists can directly test physical theories and models of phase diagrams as a function of protein concentration and crowding inside living cells (61). Such liquid-like microdomains of membraneless compartments are likely important complexes for specific reactions and cellular activities that can now be explored fully, but they will likely depend on a multitude of weak interactions to give them their liquid-like properties.

CONCLUSIONS

The strategy of physics-based disciplines like biophysics is to assume that many things do not matter. Yet time and time again, we are foiled by biology, because it appears that many more things matter than we assume, and some things that we think matter do not matter to the process we are focusing on. In this overview, I hope that the importance of the statistical and cumulative nature of molecular interactions has been conveyed. Unfortunately, many of the key players are unob-

served or unknown, acting as dark-matter species. The list of dark-matter species I present here is obviously incomplete and could include small, intracellular DNA species, short linear-interaction motifs, or other exotic phases. I encourage researchers to call out the dark-matter nature of the problems they are studying, so that the community can begin to productively move toward highlighting and making progress on these important problems.

Finally, I mention that all the processes are driven by thermal and athermal kicks and fluctuations. Biological systems have evolved to harness the energy from random fluctuations, many of which are driven by ATP/GTP-utilizing ratchets. Such thermal ratchets interact weakly with other molecules frequently enough to enable large-scale active work to be produced. Large, macromolecular complexes that perform active work in cells require the coordination of many molecular ratchets. For instance, the mitotic spindle used in cell division and the actin-myosin contraction of cell motility both couple active systems with weak interactions, intrinsically disordered proteins, and posttranslational modifications to perform large-scale and biologically essential motions.

We are beginning to illuminate many of these mechanisms, but progress is hindered by our own inability to physically visualize and mentally imagine statistical and fluctuating many-body systems. Until we can move past these barriers and visualize the dark matter acting in these roles, we will never be able to understand or engineer such structures that can utilize the fluctuations of active systems.

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