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Dynamical spectroscopy and microscopy of proteins in cells

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With a strong understanding of how proteins fold in hand, it is now possible to ask how in-cell environments modulate their folding, binding and function. Studies accessing fast (ns to s) in-cell dynamics have accelerated over the past few years through a combination of in-cell NMR spectroscopy and time-resolved fluorescence microscopies. Here, we discuss this recent work and the emerging picture of protein surfaces as not just hydrophilic coats interfacing the solvent to the protein's core and functional regions, but as critical components in cells controlling protein mobility, function and communication with post-translational modifications.

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Introduction

During the past century, research in biochemistry, molecular biology and biophysics has shown that cells are highly hierarchical, from the large organelles to individual macromolecules, metabolites and inorganic ions [1]. It is not just a structural hierarchy, but a dynamical one as well, with slow phenomena such as cell division emerging from fast phenomena such as transcriptional regulation. Dynamical protein interactions in the cell play critical roles, and the weakest and most transient of these functional essential interactions have been labeled 'quinary structure' [2].

Multiple techniques have been applied to in-cell dynamics to study how proteins transiently interact or avoid undesirable interactions in crowded environments [3], such as co-translational folding studies [4], in-cell NMR of proteins mis-matched with their cytoplasm [5], or temperature and volume jumps to control complex dissociation via heat or osmotic pressure [6]. We focus on in-cell NMR, in-cell dynamic microscopy and some of the conclusions about how proteins co-evolve to find or avoid one another in the cellular milieu.

In-cell nuclear magnetic resonance spectroscopy goes back nearly 50 years [7]. An early history [8], and two books covering efforts to 2019 are available [9,10]. Apropos this review, one of the first efforts focused on the viscosity in erythrocytes [7]. ^{15}N-enrichment of proteins overexpressed in *E. coli* cells brought the technique to the fore in the early 2000s [11]. It was soon noticed that the cellular interior attenuates rotational motion compared to buffer [12]. ^{19}F-relaxation studies showed that the attenuation arises from more than just increased viscosity — weak attractive interactions are invoked [13] — but until recently [14••] there was no model.

In-cell measurements of protein folding by microscopy originated in the early 2000s [15], and soon reached a short enough time scale to resolve kinetics [16]. Protein-binding dynamic microscopy followed [17], and current methods can compare protein dynamics among individual cells of different tissues *in vivo* [18•]. Fluorescence imaging in particular can quantify protein properties at cell-like concentrations and differentiate behavior in different organelles.

Understanding how cells work requires knowledge of structure, energetics and dynamics. Here, we focus on efforts from the past two years to find patterns in protein dynamics inside cells. Folding of globular proteins, unproductive sticking caused by attractive interactions with other macromolecules, as well as productive quinary interactions, and the dynamics of disordered proteins all depend on protein surfaces and interactions of their charge and hydrophobic patches with the surrounding matrix. Simple physico-chemical rules favoring productive interactions among the myriad possible are emerging.

Stability and dynamics in cells

NMR exploits the ability of nuclear spins to report on their chemical environment. Here, we focus on NMR experiments in solution — so called high-resolution NMR — using stable spin-1/2 nuclei that are easily enriched (e.g. ^{1}H, ^{13}C, ^{15}N) or installed as minimally

perturbing labels (i.e. ^{19}F). The strength of NMR lies in assessing kinetics on timescales from hours to nanoseconds and processes from reaction rates to internal dynamics and both translational and rotational diffusion. It is also important to bear in mind that NMR reports on ensembles of molecules.

Several challenges associated with in-cell NMR arise from its insensitivity. Acquiring high quality data even under ideal conditions, which the cellular interior is not, requires protein concentrations of 10 μM and acquisition times of seconds, or longer. Long data acquisition favors hardy cells like *Escherichia coli*, yeast or immortalized animal cells. Protein leakage from dying cells is a second problem because it is difficult to distinguish signals from the intracellular versus escaped proteins [19]. Also, non-physiologically large quantities of protein are needed to obtain high quality NMR in the cell. For example, the natural stoichiometry of any binding partner is overwhelmed by the large amounts of protein required for detection. Currently, NMR is most useful for understanding the overall influence of the cellular environment on protein properties.

It is reasonable to expect that the viscosity of the crowded cytoplasm is greater than that of buffer. This expectation is borne out by NMR studies. The effect is larger in bacteria, whose bulk protein concentration is about twice that of eukaryotic cells [20]. The increased viscosity in both instances is attributed more to attractive interactions than just the packing, including complementary charge-charge interactions, hydrogen bonding and hydrophobic interactions between the protein being studied and other macromolecules in the cell.

Strong evidence for these interactions comes from experiments that alter surface charge. The average isoelectric point of proteins in eukaryotic and prokaryotic cells is less than the physiological pH (~7.6), which means that the proteome carries a net negative charge [21•]. Increasing the positive surface charge of a macromolecule tends to slow diffusion, consistent with the idea of complementary charge-charge interactions.

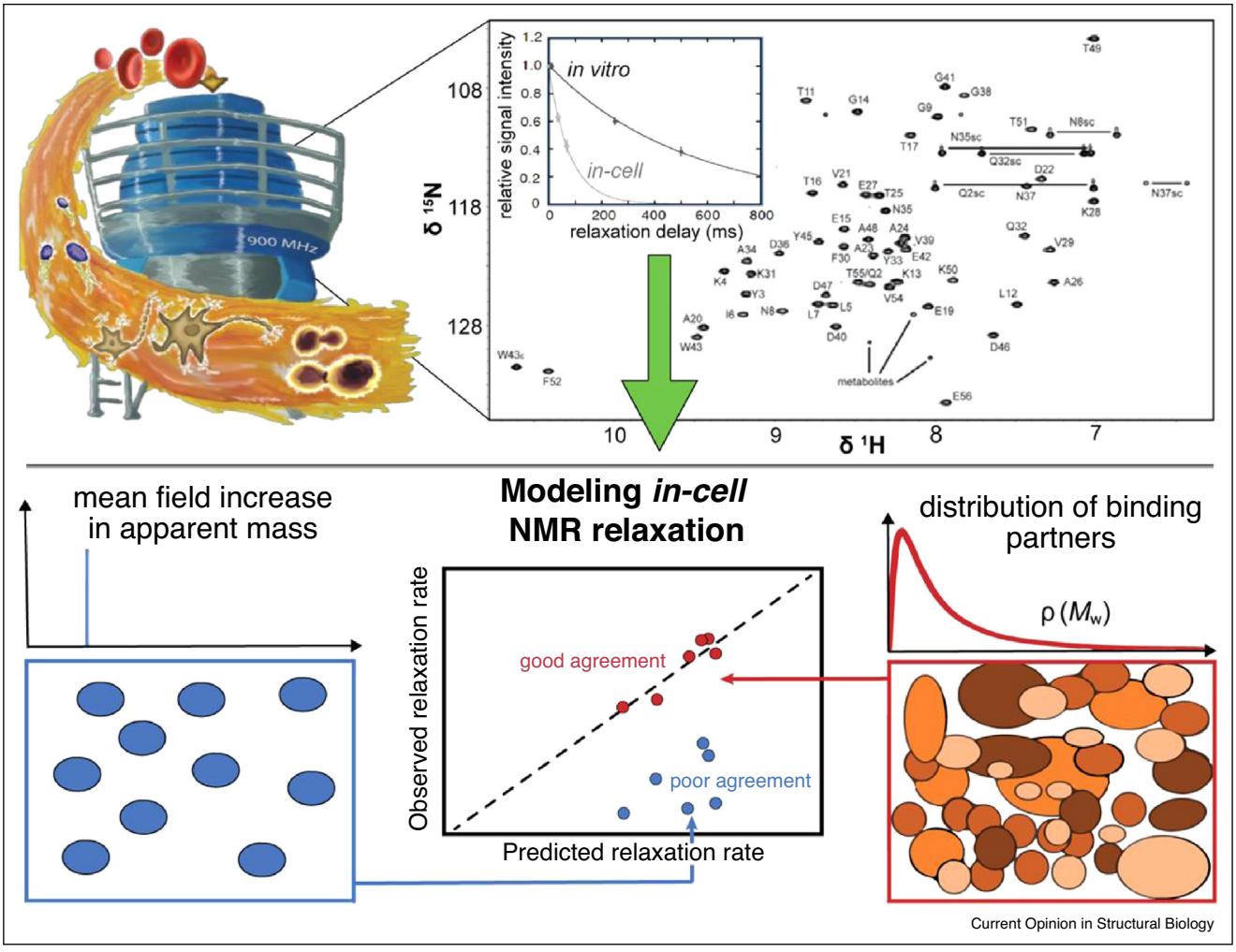
The key result from NMR-detected globular protein diffusion efforts in cells is simple to state, but until recently more difficult to explain: the increased viscosity assessed using transverse relaxation is larger than the increased viscosity as measured via longitudinal relaxation [13,22,23•]. This result has been reported several times in both *E. coli* and animal cells. Leeb *et al.* recently proposed a model that explains these observations [14••]. They posit fast exchange between the monomeric protein under study and nearby proteins in the cytoplasm (Figure 1).

Complementary to NMR, time resolved fluorescence microscopy misses the exquisite structural resolution, but can work at physiological concentrations. Choosing the label is key to avoiding label-induced sticking, which masks quinary interactions [6], or unwanted trafficking between organelles [25]. For detection, fluorescence lifetime-based probes such as FLIM and ratiometric (dual-wavelength) probes such as FRET can be combined [26]. Rational design of brighter deep-red fluorescence probes for FRET [27] or long-lived luminescence resonance energy transfer reduce background from the cell [28].

Extant techniques capable of fast dynamics fall into two categories: steady state versus perturbation. Near equilibrium, the approaches are connected by the fluctuation-dissipation theorem [29], which proves that the timescale of spontaneous fluctuations (e.g. the average rate of transitions in a single molecule experiment) is identical to the timescale of dissipation (e.g. the rate of exponential relaxation after a small perturbation). Wohland and coworkers developed fluorescence correlation spectroscopy [30] into a steady-state imaging technique [31] for whole cells and *in vivo*, looking recently at how protein diffusion gets stuck in the nucleus [32•]. The analysis of structured illumination methods is improving towards the msec regime, revealing, for example. how organelles such as the endoplasmic reticulum connect or disconnect different protein populations [33]. New perturbation techniques such as volume perturbation expand the regime from protein folding to protein interactions [6].

Recent applications illustrate the utility of dynamic microscopy (Figure 2). Ratiometric FRET of superoxide dismutase (SOD) in cells differentiating into neurons reveals that SOD is destabilized. Crowding remains similar during this process, suggesting the destabilization arises from changes in the chemical quality of the proteome [24]. In-cell binding of Hsp70 [34] and Hsc70 [35] to substrate phosphoglycerate kinase (PGK) reveals that the chaperones act differently under heat shock: the upregulated Hsp70 binds native protein on the verge of unfolding, whereas the latently expressed Hsc70 binds only at higher temperature. This difference is explained by their function: Hsc70 avoids folded proteins with transient hydrophobic exposure so as to increase its availability for other processes. Most importantly, neither protein functions as a heat shock protein *in vitro*, highlighting the importance of quinary structure for function. Other experiments on PGK [36], one of the most abundant cellular enzymes, shows that crowding produces an offset towards increased protein stability, as predicted [3], but sticking controls a trend towards increased or decreased stability. In highly crowded zebrafish eye-lens cells, sterics increases PGK stability compared to other tissues, but its folding kinetics remain similar even in this highly packed environment [18•]. As a final example, experiments reveal heterogeneous kinesin motion in cells

Figure 1



Pictorial explanation of in-cell NMR applied to sticking. In-cell NMR provides relaxation data on backbone amide groups that is used to assess models for protein diffusion. Simple mean field theories fail to reproduce the measured relaxation, but using proteomic data and a model based on fast exchange between the free-tumbling being studies and its transient complexes results in agreement [14••].

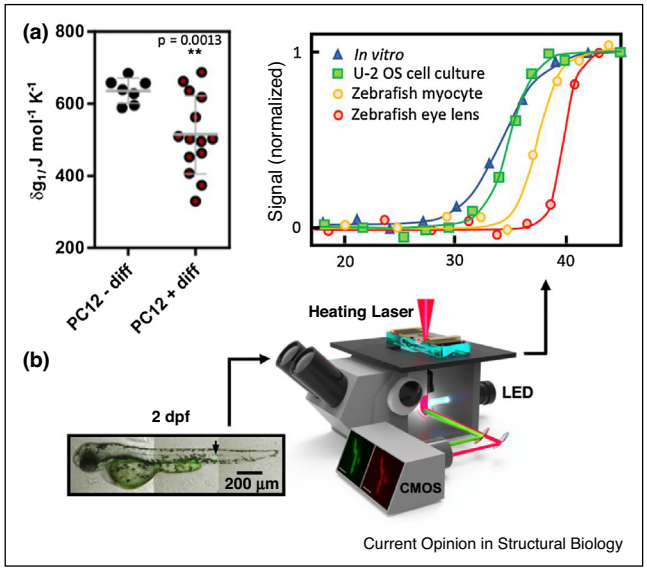
depending on the number and directionality of motors attached to its cargo [37].

Emerging rules for protein surfaces

Proteins are large for at least two reasons. First, they must precisely position functionally important residues using fairly low-resolution building blocks (amino acids) [38]. Second, they form surfaces capable of multiple useful interactions, while minimizing undesirable interactions [39]. Protein surfaces evolve with the cell just like globular protein cores evolve for folding, or active sites for catalysis. For example, flies require ATP-producing enzymes to associate with actin filaments to provide power [40], and in-cell measurements show that the cytoskeleton affects compactness of the ATP-producing enzyme PGK [41].

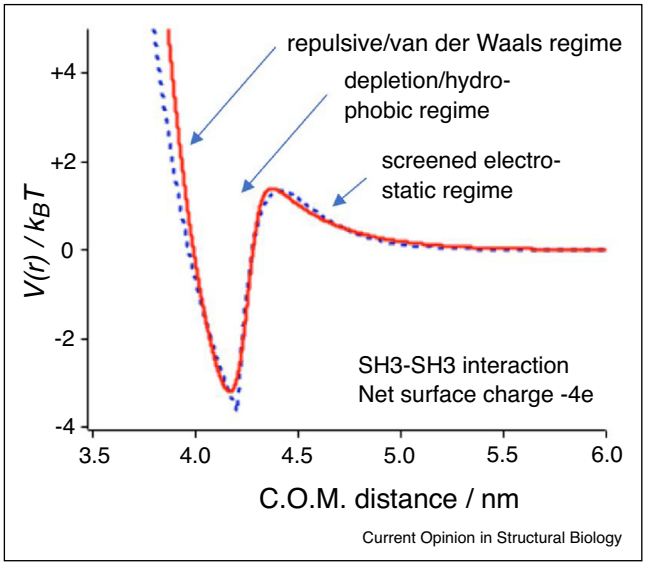
Evolution of protein surfaces and intrinsically disordered proteins (IDPs) requires descriptors more subtle than those usually associated with enzyme function (e.g. single critical sidechains). These descriptors include net charge and dipole [21•], hidden sequence complexity [42•] and hydrophobic patches [43]. Although cytoplasmic conditions differ among organisms such as prokaryotes and eukaryotes [20], NMR-analysis of genomes and protein sticking show that protein avoid clumping *via* net negative charge [21•]. For weakly interacting proteins frequently used for in-cell NMR (e.g. SOD [5]), charge and dipole are the major determinants of intracellular mobility. In addition, physiologically relevant *in vitro* environments differentially affect the diffusion of globular proteins compared to IDPs [20] with IDPs diffusing faster than expected [44]. This result has also been observed in living cells [45•].

Figure 2



(a) In non-differentiating (−diff) PC12 cells [24], the folding free energy of superoxide dismutase is more sensitive to temperature (greater δg_1) on average but has a narrower range than in differentiating cells (+diff). **(b)** Protein stability in zebrafish eye lens tissue *in vivo* is enhanced compared to muscle tissue, U-2 OS cells and buffer [18•].

Figure 3



When like charges attract: pairwise potential between equally negatively charged (−4e) SH3 domains. The blue curve is fitted from SAXS experiments to a piecewise potential of mean force, $V(r)$, in units of k_BT at room temperature [48]. The red curve is fitted to the same data using a continuous sum of repulsive exponential (e^{-br}, short range)-potentials, Lennard-Jones (medium range)-potentials and screened Coulomb (e^{-ar}/r, long range)-potentials. The distance is between the centers of mass (C.O.M.) of the two proteins. The protein center-to-center contact distance is about 3.9 nm. The transition from long-range screened Coulomb repulsion, to medium-range attraction via water depletion and hydrophobic sticking at the protein surfaces, to short-range Pauli-exclusion repulsion can be seen.

To enable more specific rejection of undesirable interactions and recognition of desirable ones, hydrophobic patches provide a shorter range interaction for quinary interaction than screened electrostatics alone [35]. Thus, charge-hydrophobic patterning is a likely candidate for a conserved feature of protein surfaces or IDPs that helps avoid general sticking in the cytoplasm while allowing recognition or binding-folding to improve cellular fitness [46]. Not coincidentally, folded proteins on average carry a net negative charge [47] even though they have positive/hydrophobic recognition motifs, whereas IDPs are as likely to carry a net positive charge to increase interaction with nucleic acids, a frequent binding partner of disordered sequences.

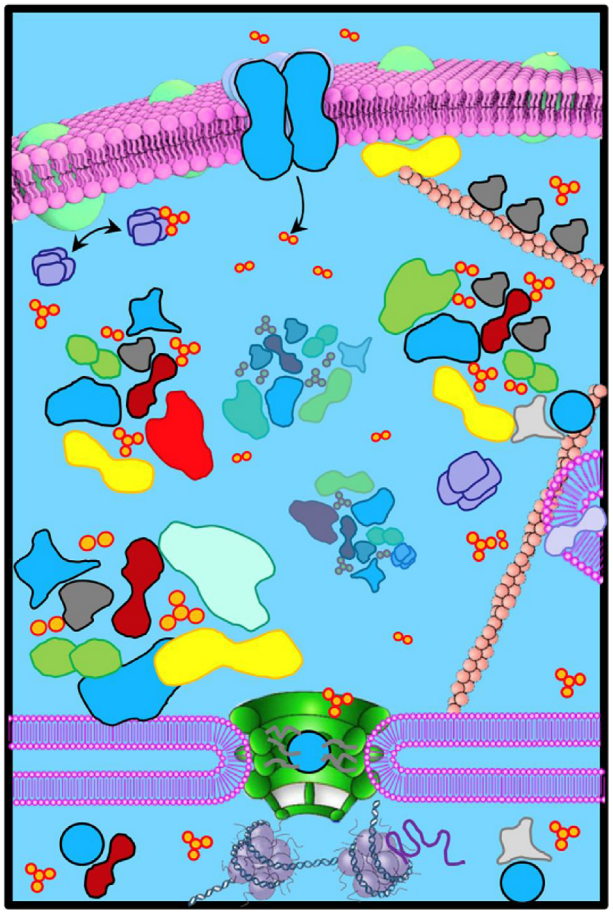
Figure 3 illustrates the different regimes based on small angle X-ray scattering measurements of how clusters of negatively charged proteins interact [48]: *via* screened repulsion with a Yukawa potential $V(r) \sim e^{-ar}/r$ at large separation, then *via* short-range attraction as the hydrophobic effects takes over (increasing water entropy by leaving the protein–protein gap and allowing hydrophobic patches to contact), and finally by repulsion again at the shortest distances where van der Waals contact becomes dominant.

Cell environment and quinary structure

Understanding how the crowded interior of cells affects globular protein stability and folding kinetics is important for two reasons. First, only the native state of a protein enzyme is active. Second, unstable proteins are prone to aggregation. Some of the first evidence that there is more to physiologically relevant crowding effects than stabilizing hard core repulsive interactions came from *in vitro* studies [49]. A few years later, these same observations were made in cell lysates [50] and then in living cells by quantifying the temperature dependence of protein stability using ^{15}N-^{1}H HSQC spectroscopy and ^{19}F NMR [51,52]. There followed several NMR-based efforts to assess folding kinetics under physiologically relevant crowded conditions [53]. The *in vitro* data show the role of attractive interactions in slowing folding and accelerating unfolding, but there are insufficient data to provide a unifying picture in cells.

Protein–protein interactions drive signal transduction, and two-thirds of disease-associated missense mutations perturb protein complexes [54,55]. ^{19}F NMR has been used to study two homodimers made by mutating the 6.2 kDa domain of protein G (GB1) [56–58]. One mutation yields a side-by-side dimer. Adding two more changes gives a domain-swapped dimer. The effects of charge

Figure 4



Quinary structures from membrane to nucleus. Shown, from the top, are signaling interactions at the membrane, metabolic enzyme complexes that increase processivity, interactions of disordered nuclear pore proteins that control nuclear access and IDPs interacting with histones.

changes in protein crowders has been studied *in vitro*. As expected, attractive interactions between the variants and crowders destabilize the complex. The side-by-side dimer has recently been examined in both *E. coli* cells and *Xenopus laevis* oocytes [59••]. The complex is more stable in both cell types than it is in buffer. Charge-charge variants were also investigated: the more anionic the homodimer, the stronger the intermolecular repulsion in cells and the more stable the complex.

Observations ranging from anomalous diffusion [60] to liquid phase-separated regions [61] show that the cytoplasm is structured on all length scales. The co-evolution of protein surfaces with the cytoplasm goes beyond the fine-tuning of solubility, sticking and function [62]. Quinary structure could also induce environment-sensitive proteins with more than onefold [63], such as lymphotactin [64], to switch folds. Similarly, IDP [20] folding is often templated by binding a specific partner, even *in vitro* [65], but many IDPs do not have known binding partners, yet they lie near the folding boundary of an Uversky plot (total charge versus sequence hydrophobicity) [66]. Shuttling between folded and extended states could be fertile ground for protein evolution in eukaryotic cells, bridging the worlds of highly variable IDPs and globular proteins. In that regard the fly-casting (binding-folding) mechanism [67] may be generalizable to multiple quinary interactions of an IDP with other proteins in the cell (Figure 4).

Outlook

Cells differ from one another in many ways: eukaryotic cells are less crowded and harbor about twice as many disordered proteins/regions as [20,68,69], and as a result protein surfaces have diverged. Specific interactions such as those between chaperones and client proteins [70], and generic ones such as charge-patterning or charge hydrophobe-patterning could drive evolution as much as the classic optimization of side chains for reactivity or core packing for foldability, particularly for disordered sequences. As discussed, tissues within a single organism can affect the same protein differently, an effect of quinary structure that may be important in development by providing even more protein variety between tissues than sequence or post-translation modification allow on their own.

New spectroscopies will enable in-cell studies of the co-evolved proteome in its natural environment. For example, *in vitro* single molecule spectroscopy of IDPs [71••] will soon be applied in cells and a simple and robust fluorecence based diffusion techniqe has been developed [72]. Correlated spectroscopies — such as fluorescence dynamics plus super resolution structure [73], will reveal structural information about 'quinary structure', complementing results from efforts including in-cell NMR that provide quantitative information about equilibrium thermodynamics, and kinetics, and more biological efforts that address fitness. The combination of these endeavors will reveal how protein homeostasis works in terms of both structure and energy in complex cellular environments, from specific molecular chaperoning to co-solutes creating a chaperoned environment.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Martin Gruebele: Conceptualization, Visualization, Writing - review & editing. **Gary J Pielak:** Conceptualization, Visualization, Writing - review & editing.

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