Submitted to: Curr. Op. Struct. Biol.

In-cell protein landscapes: making the match between theory, simulation and experiment

Gopika Gopan,[1] Martin Gruebele[1,2,3] and Meredith Rickard[1]

Theory, computation and experiment have matched up for the folding of small proteins *in vitro*, a difficult feat because folding energy landscapes are fairly smooth and free energy differences between states are small. Smoothness means that protein structure and folding are susceptible to the local environment inside living cells. Theory, computation and experiment are now exploring cellular modulation of energy landscapes. Interesting concepts have emerged, such as co-evolution of protein surfaces with their cellular environment to reduce detrimental interactions. Here we look at very recent work beginning to bring together theory, simulations and experiments in the area of protein landscape modulation, to see what problems might be solved in the near future by combining these approaches.

Addresses

[1] Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

[2] Department of Physics, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

[3] Center for Biophysics and Quantitative Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

Corresponding author: Gruebele, Martin (mgruebel@illinois.edu)

Current Opinion in Structural Biology 2020, 50:XX–XX

This review comes from a themed issue on Folding and binding Edited by Vic Arcus and Margaret Cheung

http://dx.doi.org/10.1016/j.sbi.2020.XX.XXX

Introduction

Protein folding has been studied quantitatively *in vitro* for over half a century, starting with experiments that revealed the structural, kinetic and thermodynamic principles behind folding of small proteins [1,2], soon followed by computational efforts to model folding and unfolding events [3,4]. The energy landscape theory of protein folding that crystallized in the 1990s quantified the paradigm of entropy-enthalpy compensation that allows protein folding to be such a fast (microseconds to minutes) chemical reaction

with small barriers and shallow free energy minima; yet folding is also a frustrated process because not all constraints (e.g. function vs. rapid folding) can be simultaneously optimized [5,6]. Starting about 20 years ago, *in vitro* computational and experimental efforts began to converge [7,8], culminating in computational reversible folding of a dozen small proteins [9] and more since then [10].

In contrast, a protein that is folding *in vivo* must navigate a heterogeneous environment filled with metabolites, inorganic ions, water, and many types of macromolecules. Neighboring macromolecules are considered to be particularly important because macromolecules are estimated to occupy 10-40% of a cell's volume [11], but ions and small metabolites also play a big role by screening and binding [14]. A folding protein's shallow free energy minima means that cell cycle, cell compartments, cytoskeleton and other features of the cell will modulate protein folding and protein interactions in space and time [12,13].

Theoretical and computational efforts to determine the effect of the cellular environment on proteins began in the 1980s by exploring the effects of crowding [14] and macromolecular 'sticking' [15] on protein folding and protein-protein interactions. Early efforts to simulate proteins *in vivo* modeled macromolecules as hard spheres and focused on their diffusion and neighboring interactions [16,17]. In 2010, McGuffee and Elcock simulated a sizeable segment of the *E. coli* cytoplasm using Brownian dynamics [18]. Following their work, coarse-grained cellular models and all-atom models of crowded protein solutions were developed [19,20]. In 2016, Sugita and Feig used all-atom molecular dynamics to evaluate protein stability and protein-protein interactions in a large (>100 million atom) model of a bacterial cytoplasm [21]. Since this study, the field of in-cell protein dynamics has continued to grow [22–24].

Experimental techniques to study protein folding in cells started to develop in the early 2000s. The first quantitative method to compare protein stability *in vitro* and inside cytoplasm of *E. coli* cells using mass spectroscopy was reported by the Oas group in 2001 [25]. Such early techniques were destructive to the cell by extracting its contents prior to measurement. However, with improved *in vivo* fluorescent labeling, protein stability and aggregation could be observed directly in live bacterial cells [26]. Fluorescence imaging further emerged as a powerful technique to study protein folding stability and kinetics directly in live mammalian cells [27], enabling the observation even of single molecules [28].

A number of recent reviews have covered the advances in theoretical, computational and experimental analysis of in-cell protein folding stability, diffusion and protein-protein interactions prior to the last three years [12,29–31]. The combination of improved computational capacity and increasingly sensitive experimental techniques makes the field ripe for a second theory-computation-experiment convergence *in vivo*. Here we emphasize tests of emerging ideas on protein folding and binding *in vivo* from the last two years, and discuss where the convergence can push boundaries in physics, biology and chemistry.

Theory and Computation

Theoretical studies of protein folding and binding *in vivo* have focused on three major areas: crowding, sticking, and quinary structure. Crowding (steric or repulsive interactions of neighboring macromolecules) is predicted to stabilize more compact (folded) structures. The interior of the cell also offers electrostatic and hydrophobic interactions mediated by water, ions, and other macromolecules which can destabilize proteins [32] and interfere with their diffusion around the cell [33,34]. These interactions are referred to collectively as 'sticking.' When such weak, transient interactions have evolved to benefit the organism, they are referred to as quinary structure [12,35]. The existence of sticking is yet another sign of frustration because different interactions can make conflicting demands and evolution optimizes overall cellular homoeostasis, not just any individual interaction.

The interplay of repulsive and attractive interactions has been shown experimentally to predict both stabilizing and destabilizing trends for proteins in cells [36]. Although proteins initially fold as a nascent chain during translation, their shallow energy landscapes ensure that an unfolded population is always present in the cell. For small proteins the ribosomal and unassisted folding processes may not be all that different [37]. For larger proteins, chaperones are indispensable protectors and holdases [38], reducing access to undesirable parts of the energy landscapes (e.g. aggregate global minima).

A major challenge of computational studies of crowded environments is reaching the microsecond or longer timescales necessary to fold a protein or to bring two proteins together. This is particularly challenging because early results point to slower folding in cells due to more restricted conformational dynamics [23]. To date, no protein has been folded atomistically in a cytoplasm model. Recently, we simulated multiple unfolded copies of a fast-folding WW domain for over 220 μs and observed only partial folding; instead, persistent non-native structure or even intermolecular structure [24] is formed. Additionally, coarse-grained simulations of proteins in the presence of inert crowders demonstrate the impact of crowding on structural ensembles of both folded and unfolded proteins [39,40]. In cellular simulations, the more compact ensembles are subject to a high degree of copy-to-copy variation, underscoring the importance of local environment on in-cell protein structure [21].

Multiple simulations of proteins in cell-like environments have observed excessive nonspecific protein-protein interactions and clustering [22,23,34,41]. Due to the chemical nature of protein-protein sticking, a number of forces can contribute to these interactions. Current experimental evidence emphasizes the importance of electrostatic interactions in protein-protein sticking [42,43]. Computational results also point to the importance of hydrophobic patches. While all-atom studies of concentrated protein solutions do observe protein-protein contact formation between charged residues [41,44], other studies revealed both

electrostatic and hydrophobic surface interactions in a model of the cytoplasm [23,24], or in dense homogeneous protein solutions [22].

As computational studies of proteins in the cell continue to develop, molecular dynamics force fields need to as well. Current force fields tend to over-stabilize protein-protein sticking [45]. To better simulate these environments, a number of modifications have been made to reduce sticking to the right level and better replicate experimental diffusion coefficients: using the TIP4P-D water model [22], modifying the strength of protein-water interactions [41], and using NBFIX corrections [23]. Despite these improvements, the comparison of computed and experimentally observed protein-protein sticking demonstrates that force fields developed to fold single proteins *in vitro* still need refinement to work for the cytoplasm (**Figure 1**).

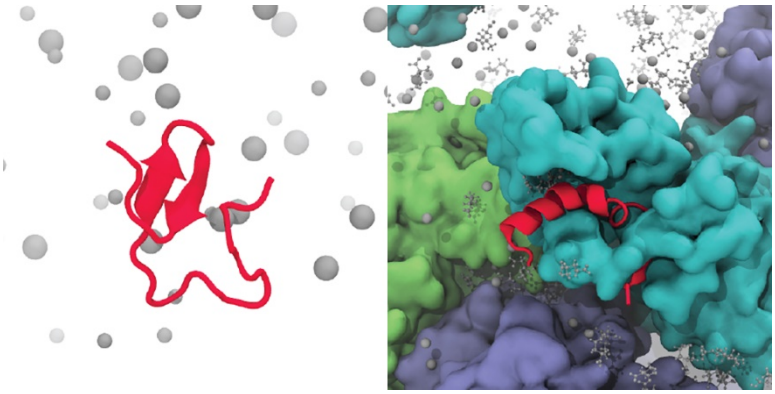

Figure 1: MD force fields' tendency to stabilize protein-protein interactions make modeling crowded environments challenging. (a) While a WW domain (red) folds easily in a waterbox simulation with ions (gray spheres = Na^+, Cl^-), (b) excessive sticking interactions between surrounding macromolecules and metabolites cause long-lived misfolded states to appear in crowded simulations (WW domain in red has formed non-native helical structure) [24].

Experiment

Protein structure, stability and function are sensitive to the local environment. Experiments under a wide range of crowding conditions have revealed great structural variety exhibited by some proteins even *in vitro* [39]. There have also been efforts to develop *in vitro* environments to better mimic the intracellular environment's interplay of crowding and sticking [46].

Crowding and sticking forces balance to control the structure of proteins inside the cell **(Figure 2)**. A recent in-cell NMR study of protein GB1 reported a significant shift in the position of a helix-loop inside the cell *vs.* the solution structure [47]. DEER measurements of calmodulin inside living cells revealed more diverse conformations adopted by the protein in comparison to *in vitro* and cell lysate measurements [48], reinforcing again the idea of copy-to-copy variation in cells [21]. Live-cell imaging of FRET-labeled PGK

revealed that crowding also causes compaction of the unfolded state in addition to stabilizing the folded state [49].

Crowding and sticking also balance to control protein stability and folding inside the cell's many different environments (**Figure 2**). Our group recently used the cell as a test tube to modulate cell volume and intracellular concentration of small solutes independently and alter the stability of an enzyme, PGK [52]. Another recent study by Gnutt *et al.* demonstrated how surface mutations that weakened interactions, presumably sticking interactions, of SOD1 with the local environment lead to stabilization of the protein inside cells [53]. Moreover, cells subject to differentiation and stress exhibit changes in proteome and quality control machinery which modulate protein folding differently compared to unperturbed cells [51]. Mosaic expression of FRET labeled-protein in zebrafish larvae enabled us to study the variability of protein stability and folding kinetics across different tissues in a living organism with single-cell resolution [54].

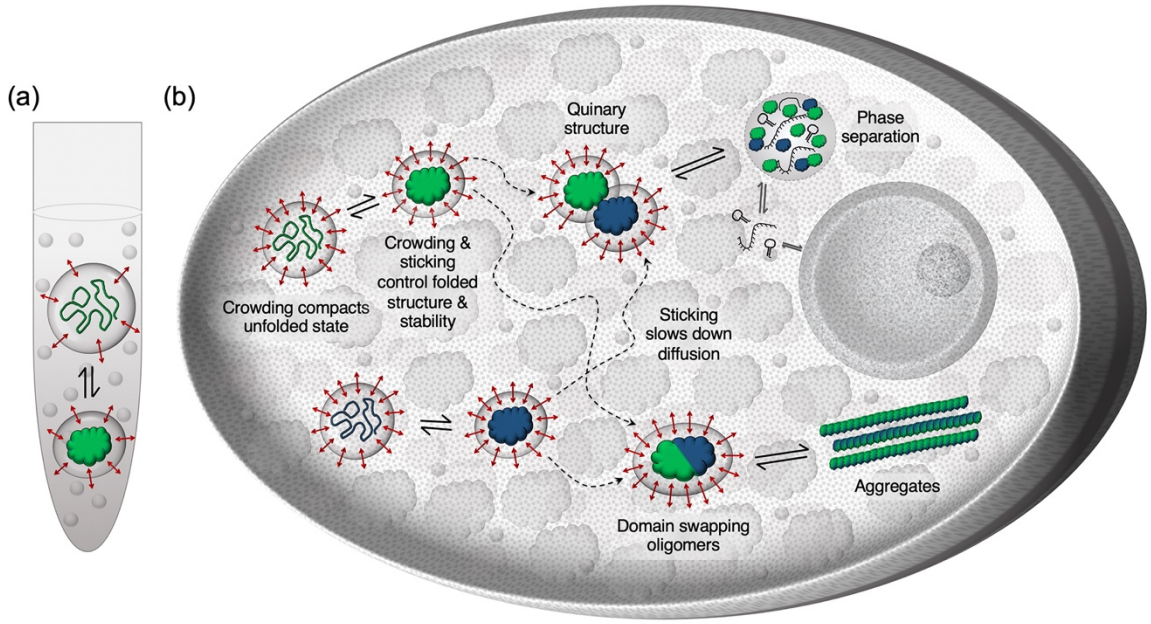

Figure 2: The complexity and impact of cellular environment on protein structure, stability and function are difficult to reproduce in a test tube. Figure summarizes the factors in play based on a few experimental results of interest. **(a)** *In vitro* experiments of proteins in dilute aqueous buffer solutions or with inert crowders often neglect the non-steric interactions between macromolecules and variability in composition inside cells [50,51]. **(b)** Effects of macromolecular crowding and sticking forces on compactness of unfolded state [49], structure and stability of folded state [52–54] and diffusion of a protein inside the cell to reach the required locations or in search of binding partners [33]. Protein-protein interactions can result in functional (quinary) or non-functional complexes/oligomers, whose shape determines the stabilization by crowding [55]. Quinary structures allow for multivalent interactions, extreme cases leading to phase separation in the crowded cellular environment [56].

In the cell, proteins interact with one another to form functional complexes. More so than strong quaternary interactions, the weakest transiently bound complexes or quinary structures are highly sensitive to variations in the local environment, useful for sensing the state of the cell and cellular signaling. As in the

case of protein folding, macromolecular crowding is expected to favor the more compact associated state of a protein complex. Recent studies on protein dimers show that the degree of this stabilization could be predicted by the shape and also by the electrostatic and hydrophobic surface properties of the dimer. Interestingly, dimers that deviate from spherical shapes show reduced stabilization in crowded environments **(Figure 2)** [55,57]. Moreover, the crucial role of the cytoplasm in maintaining weak protein-protein interactions and their function is evident from our recent study where the proper ATP-dependent heat shock function of Hsp70 on substrate PGK was enabled inside the cell, whereas *in vitro* crowded environments predominantly caused ATP-independent sticking of the unfolded substrate to the chaperone [58]. In addition, structurally similar chaperones can operate by different mechanisms inside the cell, for example binding proteins only after or also before they unfold [59].

Quinary interactions play an important role in organizing the highly crowded and complex cellular environment, sometimes even resulting in phase separation and formation of microenvironments called 'liquid droplets' (**Figure 2**). This phenomenon is mostly exhibited by disordered proteins (IDPs) that self-assemble or associate with RNA molecules [60]. The sensitivity of this association to variations in salt concentration and temperature [56] can be utilized in tuning assembly or disassembly of droplets for quantitative studies in mammalian cells. *In vivo* measurements of IDPs of opposite charge has revealed the importance of electrostatic interactions in modulating binding of nascent chains to their cellular targets [61]. Real-time in-cell NMR experiments to better understand RNA-mediated quinary interactions revealed ribosomes as major components that mediate protein quinary interactions [62,63]. The ribosome acts as a hub for nascent proteins and many factors to associate. For small proteins this may not modulate the energy landscape much [37], but for large proteins it could critically reshape the folding funnel to avoid unproductive routes.

Towards a match

Physics-based folding simulations of small proteins are encountering much success *in vitro*. In-cell, all-atom force fields still require calibration to balance self-interaction that leads to folding *vs.* electrostatic or hydrophobic sticking to the environment. To make an initial match, the key again will be timescales: as full-atom simulations of the cytoplasm ramp up towards the millisecond time, folding experiments in the cell will need to move from the current ms into the μs time scale (**Figure 3**). This will require parallelizing equilibrium techniques such as fluorescence correlation spectroscopy so that the whole cell can be imaged at once or speeding up relaxation techniques such as in-cell temperature jumps. The solution of the 'sticking' problem will also be important for simulating protein interactions because they are reduced by

excessive non-competitive binding. Current experiments do show, however, that binding in the cell can be reduced by orders of magnitude compared to *in vitro* measurements attempting to replicate physiological conditions [65], so a direct comparison of in-cell simulations and measurements will be important.

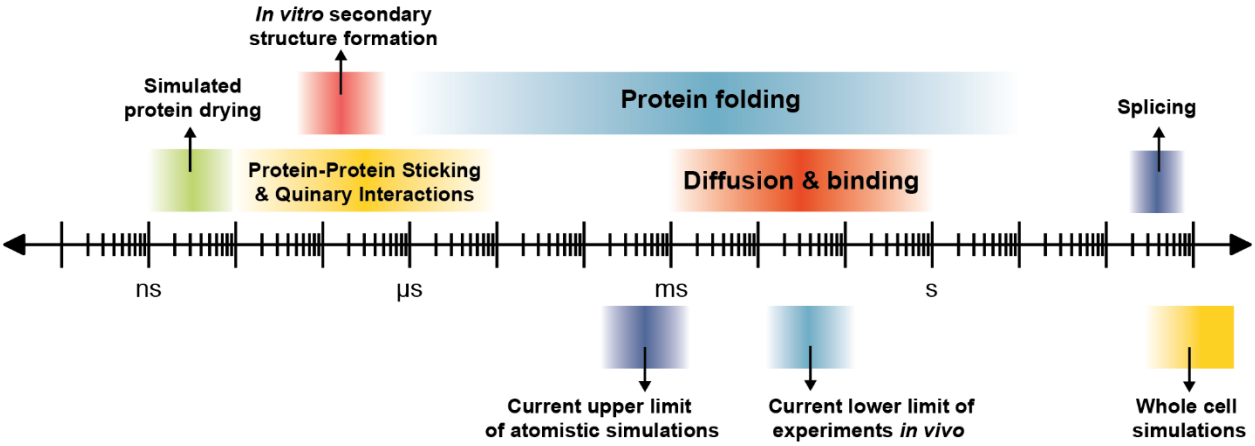

Figure 3: Timescales of notable biological processes (top) [13,23,24,64] and experimental and computational methods (bottom) [24,27,28,64]. In order to converge, the timescales of these techniques must overlap with each other and the process of interest.

In the realm of protein-protein interactions, a key question is how the cell deploys protein surfaces to avoid unwanted interactions and optimize useful interactions, as well as how the former (sticking) can evolve into the latter (quinary structure). A protein surface encodes only a finite amount of information [30] that can be used to optimize recognition of multiple binding partners and at the same time avoid non-specific binding. Overall electrostatic tuning of cytoplasmic surfaces clearly plays an important role [33], but over the next several years it will become important to understand at the residue level how protein surfaces evolve in the cell to maximize function and minimize interference.

In the meantime, 'whole cell' simulation techniques have been developed that merge coarse-grained real-space dynamics on a lattice with reaction networks for small solutes and chemical reactions that cannot be handled on the lattice [66]. This work is being extended from bacterial to eukaryotic cells [64]. As coarse-graining approaches the all-atom scale, it remains important to include reaction networks. Quantum mechanical treatments of reactions in the cell are the gold standard, but more efficient methods to combine weak interactions with chemistry (e.g. bond breaking and making in metabolism or replication) will be needed to bring a cell to life on the computer.

The ultimate goal of biomolecular folding and interaction dynamics in the cell is to merge with gene expression and replication, ribosomal structure formation [67], essential metabolism [68], and phase transitions that compartmentalize the cell with or without membranes, to produce simplified real-life and computational cells capable of replication and survival. While this is a lofty goal, the many fields of

computational and experimental biophysics that need to merge are making swift progress to put the necessary pieces in place.

Funding: This work was supported by the National Science Foundation (grant number NSF MCB 180378).

References

1. Kendrew JC, Bodo G, Dintzis HM, Parrish RG, Wyckoff H, Phillips DC: **A three-dimensional model of the myoglobin molecule obtained by x-ray analysis**. *Nature* 1958, **181**:662–666.

2. Anfinsen CB: **Principles that Govern the Folding of Protein Chains**. *Science* 1973, **181**:223–230.

3. Levitt M, Warshel A: **Computer simulation of protein folding**. *Nature* 1975, **253**:694–698.

4. Taketomi H, Ueda Y, Gō N: **Studies on Protein Folding, Unfolding and Fluctuations by Computer Simulation**. *International Journal of Peptide and Protein Research* 1975, **7**:445–459.

5. Dill KA, Chan HS: **From Levinthal to pathways to funnels**. *Nature Structural Biology* 1997, **4**:10–19.

6. Bryngelson JD, Onuchic JN, Socci ND, Wolynes PG: **Funnels, pathways, and the energy landscape of protein folding: A synthesis**. *Proteins: Structure, Function, and Bioinformatics* 1995, **21**:167–195.

7. Duan Y, Kollman PA: **Pathways to a Protein Folding Intermediate Observed in a 1-Microsecond Simulation in Aqueous Solution**. *Science* 1998, **282**:740–744.

8. Snow CD, Nguyen H, Pande VS, Gruebele M: **Absolute comparison of simulated and experimental protein-folding dynamics**. *Nature* 2002, **420**:102–106.

9. Kresten Lindorff-Larsen, Stefano Piana, Ron O. Dror, David E. Shaw: **How Fast-Folding Proteins Fold | Science**. *Science* 2011, **334**:517–20.

10. Hollingsworth SA, Dror RO: **Molecular Dynamics Simulation for All**. *Neuron* 2018, **99**:1129–1143.

11. Theillet F-X, Binolfi A, Frembgen-Kesner T, Hingorani K, Sarkar M, Kyne C, Li C, Crowley PB, Gierasch L, Pielak GJ, et al.: **Physicochemical Properties of Cells and Their Effects on Intrinsically Disordered Proteins (IDPs)**. *Chem Rev* 2014, **114**:6661–6714.

12. Wirth AJ, Gruebele M: **Quinary protein structure and the consequences of crowding in living cells: Leaving the test-tube behind**. *BioEssays* 2013, **35**:984–993.

13. Gruebele M, Dave K, Sukenik S: **Globular Protein Folding In Vitro and In Vivo**. *Annual Review of Biophysics* 2016, **45**:233–251.

14. Minton AP: **Excluded volume as a determinant of macromolecular structure and reactivity**. *Biopolymers* 1981, **20**:2093–2120.

15. Muramatsu N, Minton AP: **Tracer diffusion of globular proteins in concentrated protein solutions**. *PNAS* 1988, **85**:2984–2988.

16. Bicout DJ, Field MJ: **Stochastic Dynamics Simulations of Macromolecular Diffusion in a Model of the Cytoplasm of Escherichia coli**. *J Phys Chem* 1996, **100**:2489–2497.

17. Ridgway D, Broderick G, Lopez-Campistrous A, Ru'aini M, Winter P, Hamilton M, Boulanger P, Kovalenko A, Ellison MJ: **Coarse-Grained Molecular Simulation of Diffusion and Reaction Kinetics in a Crowded Virtual Cytoplasm**. *Biophysical Journal* 2008, **94**:3748–3759.

18. McGuffee SR, Elcock AH: **Diffusion, Crowding & Protein Stability in a Dynamic Molecular Model of the Bacterial Cytoplasm**. *PLoS Computational Biology* 2010, **6**.

19. Wang Q, Cheung MS: **A Physics-Based Approach of Coarse-Graining the Cytoplasm of Escherichia coli (CGCYTO)**. *Biophysical Journal* 2012, **102**:2353–2361.

20. Cossins BP, Jacobson MP, Guallar V: **A New View of the Bacterial Cytosol Environment**. *PLoS Comput Biol* 2011, **7**.

21. Yu I, Mori T, Ando T, Harada R, Jung J, Sugita Y, Feig M: **Biomolecular interactions modulate macromolecular structure and dynamics in atomistic model of a bacterial cytoplasm**. *eLife* 2016, **5**:e19274.

*22. Bülow S von, Siggel M, Linke M, Hummer G: **Dynamic cluster formation determines viscosity and diffusion in dense protein solutions**. *PNAS* 2019, **116**:9843–9852.

This computational paper shows how transient clustering and sticking slow down protein diffusion inside cells, using up to 3.5 million atom simulations.

23. Rickard MM, Zhang Y, Gruebele M, Pogorelov TV: **In-Cell Protein–Protein Contacts: Transient Interactions in the Crowd**. *J Phys Chem Lett* 2019, **10**:5667–5673.

24. Rickard MM, Zhang Y, Pogorelov T, Gruebele M: **Folding and sticking dynamics of a WW domain in small cytoplasm models,"** *J Phys Chem B* 2020,**124**:4732-4740.

25. Ghaemmaghami S, Oas TG: **Quantitative protein stability measurement in vivo**. *Nature Structural Biology* 2001, **8**:879–882.

26. Ignatova Z, Gierasch LM: **Monitoring protein stability and aggregation in vivo by real-time fluorescent labeling**. *PNAS* 2004, **101**:523–528.

27. Ebbinghaus S, Dhar A, McDonald JD, Gruebele M: **Protein folding stability and dynamics imaged in a living cell**. *Nature Methods* 2010, **7**:319–323.

28. König I, Zarrine-Afsar A, Aznauryan M, Soranno A, Wunderlich B, Dingfelder F, Stüber JC, Plückthun A, Nettels D, Schuler B: **Single-molecule spectroscopy of protein conformational dynamics in live eukaryotic cells**. *Nature Methods* 2015, **12**:773–779.

29. Rivas G, Minton AP: **Toward an understanding of biochemical equilibria within living cells**. *Biophys Rev* 2017, **10**:241–253.

30. Guin D, Gruebele M: **Weak Chemical Interactions That Drive Protein Evolution: Crowding, Sticking, and Quinary Structure in Folding and Function**. *Chem Rev* 2019, **119**:10691–10717.

31. Cohen RD, Pielak GJ: **A cell is more than the sum of its (dilute) parts: A brief history of quinary structure**. *Protein Sci* 2017, **26**:403–413.

32. Wennerström H, Estrada EV, Danielsson J, Oliveberg M: **Colloidal stability of the living cell. *PNAS* 2020, doi:10.1073/pnas.1914599117.

This paper shows how polyions in the cytoplasm make it a uniquely distinct environment from 'physiological' buffer, and that negative charge protein surface repulsion contributes to a stable cytoplasmic environment.

*33. Leeb S, Sörensen T, Yang F, Mu X, Oliveberg M, Danielsson J: **Diffusive protein interactions in human versus bacterial cells**. *Current Research in Structural Biology* 2020, doi:10.1016/j.crstbi.2020.04.002.

This paper compares human and bacterial proteins in *E. coli* and human cytosols to show that the mammalian cytosol is less likely to slow protein diffusion and enhance aggregation than its bacterial counterpart, making it a better evolutionary playground for protein surface mutations.

34. Nawrocki G, Karaboga A, Sugita Y, Feig M: **Effect of protein–protein interactions and solvent viscosity on the rotational diffusion of proteins in crowded environments**. *Phys Chem Chem Phys* 2019, **21**:876–883.

35. McConkey EH: **Molecular evolution, intracellular organization, and the quinary structure of proteins**. *PNAS* 1982, **79**:3236–3240.

36. Davis CM, Gruebele M: **Non-Steric Interactions Predict the Trend and Steric Interactions the Offset of Protein Stability in Cells**. *ChemPhysChem* 2018, **19**:2290–2294.

37. Guinn EJ, Tian P, Shin M, Best RB, Marqusee S: **A small single-domain protein folds through the same pathway on and off the ribosome**. *Proc Natl Acad Sci USA* 2018, **115**:12206–12211.

38. Santra M, Dill KA, de Graff AMR: **How Do Chaperones Protect a Cell's Proteins from Oxidative Damage?** *Cell Systems* 2018, **6**:743-751.e3.

39. Gasic AG, Boob MM, Prigozhin MB, Homouz D, Daugherty CM, Gruebele M, Cheung MS: **Critical Phenomena in the Temperature-Pressure-Crowding Phase Diagram of a Protein**. *Phys Rev X* 2019, **9**:041035.

40. Zegarra FC, Homouz D, Gasic AG, Babel L, Kovermann M, Wittung-Stafshede P, Cheung MS: **Crowding-Induced Elongated Conformation of Urea-Unfolded Apoazurin: Investigating the Role of Crowder Shape in Silico**. *J Phys Chem B* 2019, **123**:3607–3617.

41. Nawrocki G, Wang P, Yu I, Sugita Y, Feig M: **Slow-Down in Diffusion in Crowded Protein Solutions Correlates with Transient Cluster Formation**. *J Phys Chem B* 2017, **121**:11072–11084.

42. Cohen RD, Pielak GJ: **Electrostatic Contributions to Protein Quinary Structure**. *Journal of the American Chemical Society* 2016, **138**:13139–13142.

43. Mu X, Choi S, Lang L, Mowray D, Dokholyan NV, Danielsson J, Oliveberg M: **Physicochemical code for quinary protein interactions in Escherichia coli**. *PNAS* 2017, **114**:E4556–E4563.

44. Nawrocki G, Im W, Sugita Y, Feig M: **Clustering and dynamics of crowded proteins near membranes and their influence on membrane bending. *Proc Natl Acad Sci USA* 2019, **116**:24562–24567.

This simulation paper finds a crowding-depleted zone near the cell membrane, where the occasional passer-by protein can diffuse more rapidly than elsewhere in the cytosol, perhaps enhancing search for interaction partners near the cell membrane.

45. Petrov D, Zagrovic B: **Are current atomistic force fields accurate enough to study proteins in crowded environments?** *PLoS computational biology* 2014, **10**:e1003638–e1003638.

46. Davis CM, Deutsch J, Gruebele M: **An in vitro mimic of in-cell solvation for protein folding studies**. *Protein Science* 2020, **29**:1060–1068.

47. Tanaka T, Ikeya T, Kamoshida H, Suemoto Y, Mishima M, Shirakawa M, Güntert P, Ito Y: **High-Resolution Protein 3D Structure Determination in Living Eukaryotic Cells**. *Angewandte Chemie International Edition* 2019, **58**:7284–7288.

48. Dalaloyan A, Martorana A, Barak Y, Gataulin D, Reuveny E, Howe A, Elbaum M, Albeck S, Unger T, Frydman V, et al.: **Tracking Conformational Changes in Calmodulin in vitro, in Cell Extract, and in Cells by Electron Paramagnetic Resonance Distance Measurements. *ChemPhysChem* 2019, **20**:1860–1868.

This paper shows experimentally that calmodulin inside cells samples a much broader range of open conformations, indicative of sticking to in-cell components, and in sharp contrast with *in vitro* and cell extract results.

*49. Wang Y, Sukenik S, Davis CM, Gruebele M: **Cell Volume Controls Protein Stability and Compactness of the Unfolded State**. *J Phys Chem B* 2018, **122**:11762–11770.

This paper shows that cell volume regulated by osmotic pressure controls the compactness of a probe protein unfolded state more so than it affects protein melting temperature.

50. Caitlin M. Davis, Martin Gruebele: **Non-Steric Interactions Predict the Trend and Steric Interactions the Offset of Protein Stability in Cells**. *ChemPhysChem* [date unknown], **19**:2290–2294.

51. Gnutt D, Sistemich L, Ebbinghaus S: **Protein Folding Modulation in Cells Subject to Differentiation and Stress**. *Front Mol Biosci* 2019, **6**.

52. Sukenik S, Salam M, Wang Y, Gruebele M: **In-Cell Titration of Small Solutes Controls Protein Stability and Aggregation**. *J Am Chem Soc* 2018, **140**:10497–10503.

53. Gnutt D, Timr S, Ahlers J, König B, Manderfeld E, Heyden M, Sterpone F, Ebbinghaus S: **Stability Effect of Quinary Interactions Reversed by Single Point Mutations. *J Am Chem Soc* 2019, **141**:4660–4669.

This experimental study shows that some surface mutations of superoxide dismutase can stabilize the protein inside mammalian cells compared to *in vitro*, whereas most mutations do not.

*54. Feng R, Gruebele M, Davis CM: **Quantifying protein dynamics and stability in a living organism**. *Nature Communications* 2019, **10**:1–7.

In this experimental study single cells of a single tissue type in zebrafish express fluorescent-labeled proteins to show how tissue-dependent crowding affects local protein stability.

*55. Yang Y, Chen S-N, Yang F, Li X-Y, Feintuch A, Su X-C, Goldfarb D: **In-cell destabilization of a homo-dimeric protein complex detected by DEER spectroscopy**. *bioRxiv* 2020, doi:10.1101/2020.03.27.011510.

By Electron paramagnetic resonance at three sites this study shows that a homo-dimeric protein dissociates much more easily inside human cells and in the crowding agent Ficoll, contrary to expectations from simple crowding.

56. Schuster BS, Reed EH, Parthasarathy R, Jahnke CN, Caldwell RM, Bermudez JG, Ramage H, Good MC, Hammer DA: **Controllable protein phase separation and modular recruitment to form responsive membraneless organelles**. *Nature Communications* 2018, **9**:1–12.

57. Guseman AJ, Goncalves GMP, Speer SL, Young GB, Pielak GJ: **Protein shape modulates crowding effects**. *PNAS* 2018, **115**:10965–10970.

*58. Guin D, Gelman H, Wang Y, Gruebele M: **Heat shock-induced chaperoning by Hsp70 is enabled in-cell**. *PLoS One* 2019, **14**.

This in-cell fluorescence microscopy study shows that Hsp70 binds a client pre-emptively before the midpoint of the unfolding transition is reached in-cell, whereas the chaperone does not work at all *in vitro* even in presesence of Hsp40 and ATP.

59. Guin D, Gruebele M: **Chaperones Hsc70 and Hsp70 Bind to the Protein PGK Differently inside Living Cells**. *J Phys Chem B* 2020, **124**:3629–3635.

60. Lin Y, Protter DSW, Rosen MK, Parker R: **Formation and Maturation of Phase-Separated Liquid Droplets by RNA-Binding Proteins**. *Molecular Cell* 2015, **60**:208–219.

61. Marino J, Buholzer KJ, Zosel F, Nettels D, Schuler B: **Charge Interactions Can Dominate Coupled Folding and Binding on the Ribosome**. *Biophysical Journal* 2018, **115**:996–1006.

62. Breindel L, DeMott C, Burz DS, Shekhtman A: **Real-Time In-Cell Nuclear Magnetic Resonance: Ribosome-Targeted Antibiotics Modulate Quinary Protein Interactions**. *Biochemistry* 2018, **57**:540–546.

63. Breindel L, Yu J, Burz DS, Shekhtman A: **Intact ribosomes drive the formation of protein quinary structure**. *PLOS ONE* 2020, **15**:e0232015.

64. Ghaemi Z, Peterson JR, Gruebele M, Luthey-Schulten Z: **An in-silico human cell model reveals the influence of spatial organization on RNA splicing**. *PLoS Comput Biol* 2020, **16**:e1007717.

65. Davis C, Guzman I, Gnutt D, Gruebele M: **How much do non-specific interactions in the cell reduce protein-RNA binding affinity compared to in vitro?** *in preparation* 2020,

66. Chew W-X, Kaizu K, Watabe M, Muniandy SV, Takahashi K, Arjunan SNV: **Reaction-diffusion kinetics on lattice at the microscopic scale**. *Phys Rev E* 2018, **98**:032418.

67. Earnest TM, Lai J, Chen K, Hallock MJ, Williamson JR, Luthey-Schulten Z: **Toward a Whole-Cell Model of Ribosome Biogenesis: Kinetic Modeling of SSU Assembly**. *Biophysical Journal* 2015, **109**:1117–1135.

68. Breuer M, Earnest TM, Merryman C, Wise KS, Sun L, Lynott MR, Hutchison CA, Smith HO, Lapek JD, Gonzalez DJ, et al.: **Essential metabolism for a minimal cell**. *eLife* 2019, **8**:e36842.