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Structural Biology outside the box — inside the cell

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Recent developments in cellular cryo-electron tomography, in-cell single-molecule Förster resonance energy transfer-spectroscopy, nuclear magnetic resonance-spectroscopy and electron paramagnetic resonance-spectroscopy delivered unprecedented insights into the inner workings of cells. Here, we review complementary aspects of these methods and provide an outlook toward joint applications in the future.

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Introduction

The aim of this review is to describe complementary approaches for studying protein structure and function directly in cells. Indeed, we believe that much of the future of Structural Biology lies in cells and critically depends on our ability to integrate the cellular environment as a key parameter in our investigations. This requires new and improved methods and experimental rationales, which we shall discuss here. In the following paragraphs, we outline how the combined use of cellular cryo-electron tomography (ET), single-molecule Förster resonance energy transfer (FRET), in-cell nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopy paves the way for future applications in Cellular Structural Biology [1,2].

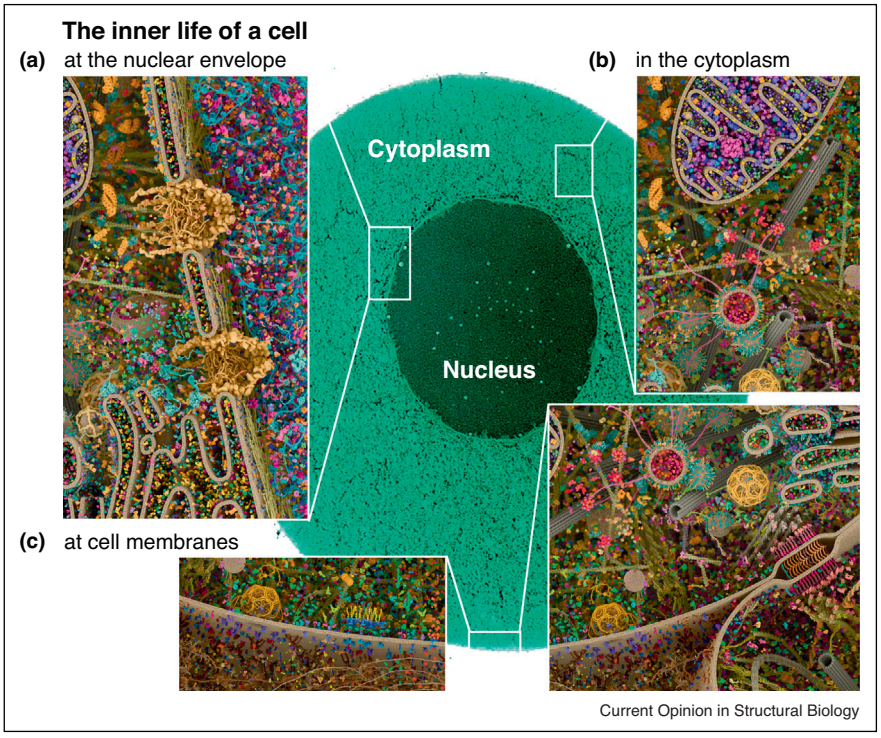
The cellular interior represents an exquisitely complex and very crowded environment with little resemblance to the isolated experimental setups that we typically employ to study proteins (Figure 1). Instead, it imposes unique physicochemical constraints on its components, governing their properties over multiple length-scales and timescales [3]. In addition, every cell type harbors a specific biological makeup, both in composition and activity, which adds another level of confounding regulatory complexity. The sum of these factors gives rise to the collective behaviors of the systems under investigation, manifested in the phenotypes that we wish to ultimately understand. For these reasons, major efforts are undertaken to develop tools to directly study biomolecules in their native cellular settings, that is, *in situ*. Recent advances in many of these methods achieved impressive degrees of robustness and sophistication, which, in turn, produced exciting new insights into the structures and functions of proteins in intact cells. They also established the experimental reference frame for combined applications in the future.

Cellular cryo-ET

We begin our journey at the nanometer to subnanometer resolution level and introduce cryo-electron tomography (cryo-ET) as a tool to explore the molecular architecture of macromolecular protein complexes in their native cellular settings. Spurred by recent breakthroughs in detector technologies [4], image processing and 3D reconstruction [5], and sample preparation [6], cryo-ET is joining single-particle electron microscopy (EM) at the center stage of the ongoing 'revolution in resolution' [7]. Owing largely to its integrative role between molecular and cellular Structural Biology [8], cryo-ET is poised to enact a leading role in future studies addressing the molecular sociology of cells in close-to-atomic detail [9•].

The general principle of cryo-ET is to record 2D projection images of a vitrified sample along defined intervals of tilt angles, typically −60° to +60°. From these projections, 3D volumes or tomograms are constructed. Tomograms are then analyzed by segmentation of specific elements of interest, which, in the case of cellular specimens, may contain different biological structures such as macromolecular machines, cytoskeletal filaments, membrane compartments or entire organelles. By extracting and combining multiple instances of such components in a procedure called subtomogram averaging [10•], medium to high-resolution structures can be obtained. In this way, experimental single particle information is turned into representative ensemble descriptions of the structures under investigation. Alternatively, known high-resolution structures can be fitted into individual tomograms to produce realistic models of the spatial organization of the intracellular space. Several technical advances propelled cellular cryo-ET applications in recent years, with the introduction of direct detector devices (DDD), that

Figure 1



Cellular architecture and composition. Artistic overview of cellular components **(a)** at the nuclear envelope, **(b)** the cytoplasm and **(c)** cell membranes, for which we discuss experimental results from recent *in situ* studies in the different sections of the review. The background picture depicts a low-resolution light microscopy image of a thin section through a giant *Xenopus* oocyte stained with hematoxylin–eosine (HE) and inverse colored for clarity. It serves to illustrate the confounding complexity of the cytoplasm already visible at low magnification, with the cell nucleus at the center of the image. Close-up images of molecular landscape scenarios kindly provided by Gaël McGill at Digizyme.

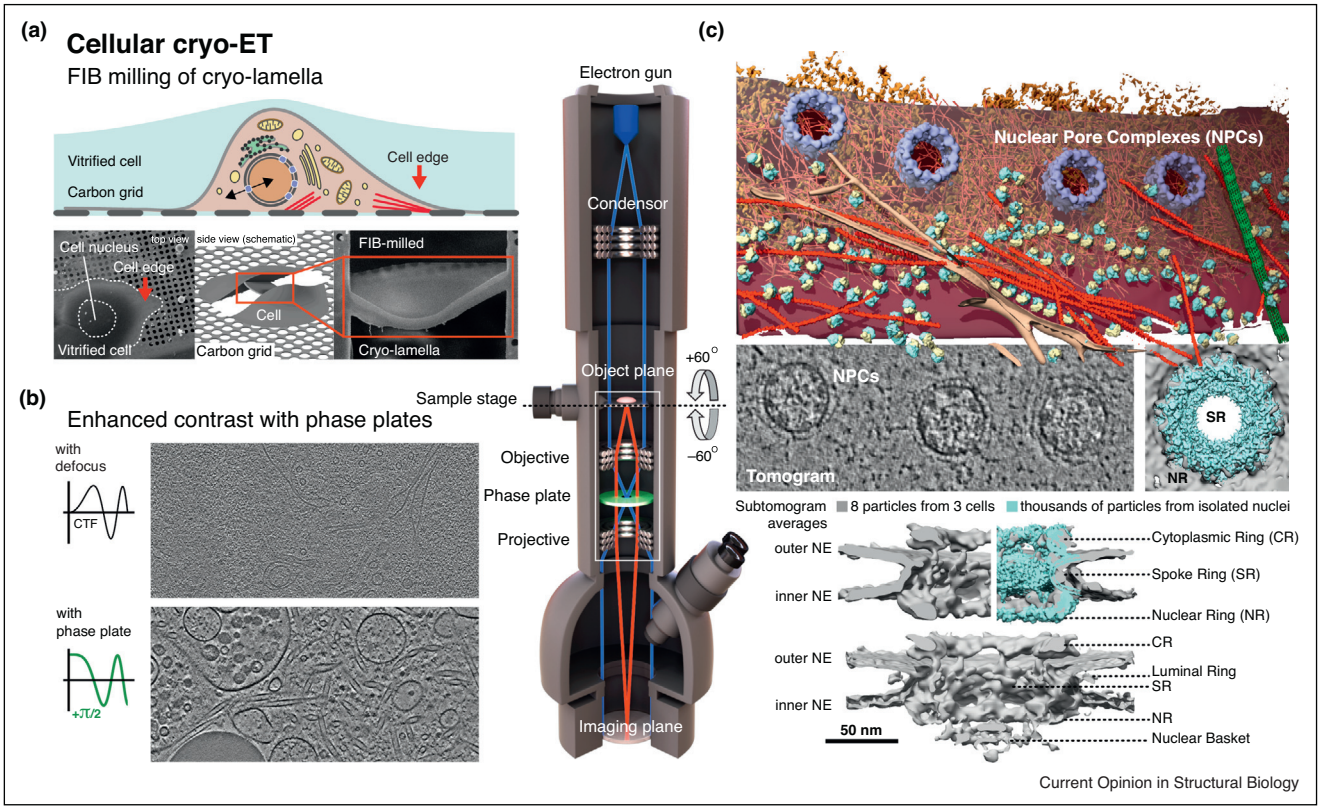
is, direct electron detectors as one key contribution. In contrast to previous charge-coupled devices (CCDs), DDDs exhibit greater sensitivity in terms of detective quantum efficiency (DQE) and higher frame-rates of image acquisition for fast recordings of multiple frames. Thus, they enable procedures to correct for specimen motions and drifts in post-acquisition image processing. Both sensitivity and readout speed produce higher resolution in the respective 3D reconstructions.

Cellular cryo-ET is generally limited by how far electrons can penetrate through a sample. This restricts *in situ* applications to specimens that are less than ~0.5 μm thick i.e. small prokaryotic cells or peripheral regions of intact eukaryotic cells. To study bulkier objects, cellular sections have to be prepared by either mechanical trimming, that is, cryo-sectioning of frozen hydrated specimens (CEMOVIS), or by focused ion-beam (FIB) milling of vitrified cell samples [11–13] (Figure 2a). Both procedures create thin slices of the cytoplasm in which a small portion of the intracellular volume is preserved. The preparation of cryo-lamellae by FIB milling is emerging as the method of choice for cellular cryo-ET applications, largely because of the absence of compression artifacts and the ability to target and manipulate samples directly on the respective EM grids.

Phase contrast is another important parameter in cryo-ET experiments of unstained biological samples. Traditionally, it is obtained by recording 2D projections slightly out-of-focus to generate defocus phase contrast. This creates several unwanted effects due to oscillations of the resulting phase contrast transfer function (CTF) that need to be dealt with. The recent development of a Volta-potential phase plate enabled researchers to overcome this problem [14,15] (Figure 2b). Indeed, future applications will greatly benefit from cryo-ET setups that combine FIB milling, the use of direct electron detectors and Volta phase plates [16], as highlighted by studies of chloroplasts and Golgi ultrastructures in vitreous *Chlamydomonas* cells [17,18], 26S proteasomes in intact hippocampal neurons [19], organelle organization in *C. elegans* embryos and adult worms [20,21] and of the translocon-associated protein complex (TRAP) at the ER of human fibroblasts [22].

One particularly appealing example to illustrate how these technical advances shaped our understanding of

Figure 2



Cellular cryo-ET. **(a)** A schematic cross section of an adherent vitrified eukaryotic cell is shown on the top. Organelles and proteins such as the nuclear pore complex (NPC, purple) connecting the cytoplasm and nucleoplasm are indicated. Cells or cellular areas thicker than 0.5–1 μm are non-transparent to medium voltage (≤300 kV) electrons, restricting tomographic studies to thin peripheral regions (red arrows). Thicker samples are 'thinned', by focused ion beam (FIB) milling (bottom panel). A top-view scanning electron microscope (SEM) image of a vitrified HeLa cell is shown on the left. FIB milling from the top and the bottom produces cryo-lamellae of uniform thickness (schematic, middle image). Resulting lamellae as seen by SEM are shown on the right. **(b)** Conventional phase contrast is induced by negative defocusing (underfocusing). Structural features remain mostly invisible (top panel; *x–y* slice of a defocused tomogram). Phase plates introduce a phase shift between the scattered and non-scattered waves at the microscope's diffraction plane resulting in greatly improved contrast (bottom panel; *x–y* slice of a Volta phase plate (VPP) tomogram). **(c)** Tomogram analysis; surface-rendered visualization of a tomographic volume, displaying the nuclear envelope, underlying lamin meshwork, nuclear pore complexes (NPCs), microtubules, actin filaments and ribosomes identified by template matching of a HeLa cell. A tomographic slice containing individual NPCs is shown below. The image at the far right displays a bottom-up view of subtomogram averages of HeLa NPCs in gray and the high-resolution average of isolated HeLa NPCs determined by von Appen *et al.* [28] in cyan. Below, individual subtomogram averages (gray) are shown perpendicular to the nucleo-cytoplasmic axis, with Nup densities of inner and outer ring structures labeled. A superposition with the von Appen structure (cyan) is depicted on the right. The final tomographic reconstruction of average HeLa NPC structures is shown at the bottom. The positions of inner and outer nuclear envelope (NE) membranes are indicated.
Adapted from Mahamid *et al*. [31••].

biological processes carried out by large, membrane-embedded macromolecular assemblies is the compendium of recent cryo-ET work on the nuclear pore complex (NPC) (Figure 2c). Made up of multiple copies of about 30 different proteins called nucleoporins (Nups), this ~120 megadalton complex allows the passage of proteins and RNA between the cytoplasm and nucleus in every eukaryotic cell. Spurred by initial results obtained with isolated *Xenopus laevis* nuclear envelope membranes [23] and intact *Dictyostelium discoideum* nuclei [24,25], recent breakthroughs extended these efforts to different functional states of *Xenopus* NPCs [26], purified human nuclear envelopes containing native NPCs [27–29], and structural NPC studies in intact human U2OS [30] and HeLa cells [31••]. Whereas these studies provided unprecedented structural information about the rigid parts of the NPC, such as the Nup scaffolds forming the outer and inner rings of the complex [28,29], they also revealed the inherent difficulties to resolve dynamic regions of the complex, including cytoplasmic Nup filaments, the nucleoplasmic 'basket' structure, and the central 'plug' region harboring phenylalanine- and glycine-rich (FG) Nup's that make up the selectivity barrier for targeted transport processes (see Figure 1a for an artistic overview). Such high-mobility parts

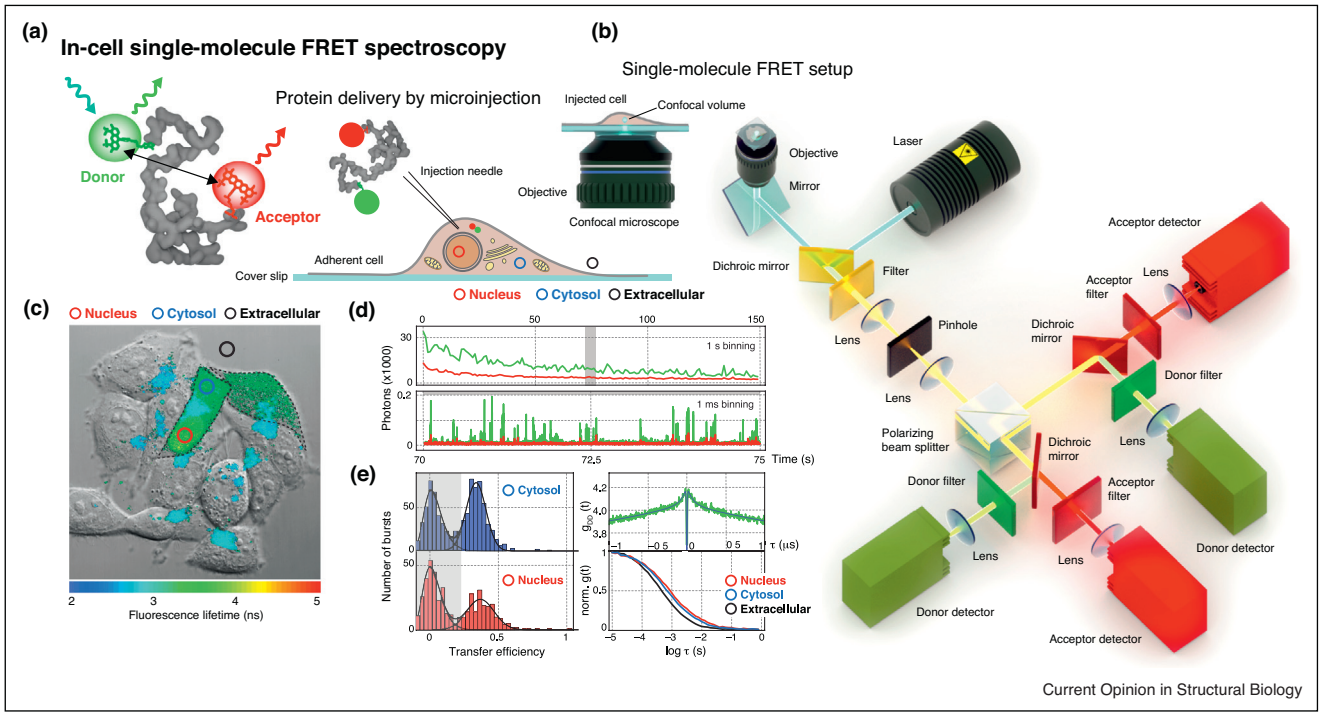
in large macromolecular assemblies often exist in multiple interconverting or disordered conformations that remain invisible due to 'freezing-out' of inhomogeneous structural populations and sample averaging. In such instances, alternative methods such as the ones we discuss next, offer invaluable complementary insights. Indeed, single-molecule FRET [32] and NMR spectroscopy [33,34] had been used previously to study FG-Nups *in vitro* and in intact bacterial cells, thus indicating the suitability for joint cryo-ET applications in the future.

In-cell single-molecule FRET

We continue with single-molecule spectroscopy, which has become a mature tool for probing biomolecular structure and dynamics over the past 20 years. FRET provides a 'spectroscopic ruler' to measure distances and distance dynamics between fluorescent donor and acceptor dyes attached at specific positions in biomolecules [35,36] (Figure 3a). Resonance energy transfer between the two dyes is highly distance-dependent and can be read out via the photons they emit upon donor excitation. By counting the numbers of donor and acceptor photons or quantifying changes in their fluorescence lifetimes, energy transfer efficiencies are determined and converted into distance information. Importantly, and in contrast to cryo-ET methods, FRET measurements are carried out at ambient temperature and in solution, hence, under truly physiological conditions. However, FRET experiments require the attachment of bulky dye pairs to the biomolecules of interest, which may interfere with intracellular localization, dynamics and structure (e.g. due to hydrophobic effects or charge interactions) and thus necessitate careful control measurements.

Research areas in which single-molecule FRET has proven particularly valuable range from the mechanisms of molecular machines [37,38] to protein-nucleic acid interactions [39,40], enzymatic reactions [41,42], and

Figure 3



In-cell single-molecule FRET spectroscopy. **(a)** Protein molecules labeled for FRET with donor and acceptor fluorophores are microinjected into adherent eukaryotic cells and **(b)** probed by confocal single-molecule spectroscopy. The essential optical components for four-channel detection are illustrated for time-correlated single-photon counting with sensitivity for emission wavelength (donor and acceptor emission), polarization (polarizing beam splitter) and correlation analysis with picosecond time resolution. **(c)** The success of microinjection can be monitored by confocal fluorescence microscopy, here illustrated by a fluorescence lifetime image overlaid with a differential interference contrast image. In this example, injected cells (green) exhibit longer-lifetime emission from FRET-labeled molecules than from autofluorescence (light blue). Red, blue, and black circles indicate measurements in the nucleus, cytosol, and extracellular medium, respectively (see also panel **(e)**). **(d)** Examples of fluorescence time-traces recorded in the cytosol. Photobleaching results in characteristic intensity decays on the timescale of minutes (1-s binning). With 1-ms binning, fluorescence bursts of donor (green) and acceptor (red) photons are visible. (e) Examples of FRET efficiency histograms acquired in the cytosol and nucleus, and correlation analysis on the nanosecond to millisecond timescale that enables distances, distance dynamics, and translational diffusion to be probed in live cells.
Adapted from König *et al.* [56••].

protein and RNA folding [36,43]. By eliminating drawbacks of experimental ensemble averaging, single-molecule FRET can often resolve structural and dynamic heterogeneity inaccessible in ensemble methods such as NMR and EPR. In this manner, different conformations are detected as subpopulations with different transfer efficiencies and, thus, distances between fluorophores, even when they coexist in solution. In addition, interconversion dynamics can be extracted on timescales from nanoseconds to hours and beyond. Specific experimental strategies have been developed to access different time regimes, including the detailed analysis of photon statistics from freely diffusing molecules, the recording of fluorescence trajectories from immobilized molecules, and the use of microfluidic mixing devices for non-equilibrium measurements [36]. Additional efforts have led to the development of techniques to extract precise and accurate structural information from single-molecule FRET experiments [44–47]. When results from multiple samples with FRET pairs at different positions are combined, the conformations and arrangements of large and even dynamic complexes can be elucidated. However, the vast majority of single-molecule FRET experiments has been limited to samples *in vitro*, albeit on some remarkably complex, reconstituted systems [35].

In standard fluorescence microscopy, FRET is very popular for characterizing biomolecular interactions, most commonly employing the green fluorescent protein (GFP) and its variants [48]. However, the relatively large size and suboptimal photophysical and photochemical properties of these genetically encoded labels have limited their use for quantitative structural studies with single-molecule detection. Despite these drawbacks, recent ensemble FRET approaches produced novel insights into the architecture of cytoplasmic protein complexes [49,50•], the physicochemical effects of intracellular macromolecular crowding [51,52], and into the folding and stability of proteins within and across different cell types [53,54]. By contrast, single-molecule experiments for studying protein structures and dynamics in intact cells had remained challenging. Whereas single-molecule detection in or on cell membranes was achieved early on [55], in-cell single-molecule FRET has become available only during the past five years, due to several experimental requirements that had to be satisfied simultaneously [56••,57]. In particular, these are: first, the necessity to minimize cellular auto-fluorescence; second, the need for a robust and reproducible method to deliver the sub-nanomolar sample concentrations required for single-molecule detection, while; third, ensuring cell survival; fourth, enabling prompt single-molecule FRET measurements to avoid intracellular sample degradation; and fifth, data analysis tools that make optimal use of the limited reservoir of fluorescent molecules per cell. Over the past few years, many of these challenges were overcome.

In 2010, Sakon and Weninger established single-cell microinjection as a suitable tool to deliver fluorophore-labeled proteins into intact mammalian cells for single-molecule FRET measurements [58••] (Figure 3a). Thereby, they solved the problem of efficient intracellular sample delivery without compromising cell viability. Moreover, they demonstrated the use of FRET dyes with excitation maxima above the wavelength range dominated by cellular auto-fluorescence, the major impediment to in-cell single-molecule detection. These advances enabled them to study the conformational changes of the membrane-fusion protein SNAP-25 in response to SNARE complex assembly in mammalian BS-C-1 epithelial kidney and PC-12 neuroendocrine cells. By optimizing sample delivery and its integration with confocal single-photon counting, König *et al.* succeeded in measuring the dimensions and sub-microsecond chain dynamics of prothymosin α in intact HeLa cells, demonstrating that the disordered state of this protein is preserved in a human intracellular environment [56••]. They further delineated the thermodynamic profile of heat- and cold-denaturation of the marginally stable protein frataxin in live cells and determined the millisecond folding kinetics of the protein G B1 domain under physiological cell conditions using recurrence analysis, a technique that benefits from the confinement and reduced translational diffusion that fluorescently labeled molecules experience in cells [59]. These results highlight another important aspect of in-cell single-molecule FRET measurements: the ability to obtain quantitative information about intracellular protein dynamics (see below).

For the generation of in-cell FRET samples in bacteria, protein delivery by microinjection is not suitable owing to the much smaller size of prokaryotic cells. Instead, Fessl *et al.* transfected fluorescently labeled DNA into *Escherichia coli* via the classical heat-shock procedure, yielding suitable samples for in-cell single-molecule FRET measurements [60]. Kapanidis and colleagues established electroporation for the delivery of fluorescently labeled proteins and DNA into bacterial cells [61••] and successfully employed this approach to investigate DNA replication, transcription and repair [62], underscoring the emergence of in-cell single-molecule FRET approaches from classical *in vitro* applications. Based on these developments, a comprehensive experimental framework to characterize the structural and dynamic properties of proteins by in-cell single-molecule FRET applications is now in place. However, further efforts are needed to resolve persistent problems of cellular autofluorescence, photobleaching and the poor overall photon statistics of intracellular FRET probes.

Figure 3 illustrates confocal single-molecule FRET measurements in mammalian cells, including the key optical components required for time-correlated single-photon

counting and correlation analysis with wavelength- and polarization-sensitive fluorescence detection and picosecond time resolution (Figure 3b). Protein microinjection (Figure 3a) is carried out on individual cells and verified by fluorescence intensity or lifetime imaging (Figure 3c). Single-molecule FRET measurements are performed until all delivered protein molecules are bleached, which typically occurs within a few minutes (Figure 3d). Injection and measurements are then repeated on another cell. Despite these short acquisition times, single-cell transfer efficiency histograms provide sufficient statistics to derive information about fluorophore–fluorophore distances and the distribution of conformational subpopulations (Figure 3e). Cell- or organelle-specific protein properties can be delineated from measurements in different cell types or in different cellular regions, for instance the cytosol versus the cell nucleus, [56••]. Information on equilibrium dynamics is available from photon statistics, especially via correlation analysis: Examples extend from translational diffusion times in the range of milliseconds [63] to fast conformational motions on the sub-microsecond timescale, as observed for global chain dynamics of intrinsically disordered proteins [56••] (Figure 3d).

The unique ability of single-molecule FRET spectroscopy to resolve structural and dynamic protein properties with high time resolution comes at the expense of structural detail and spatial resolution within the cell. In this regard, cellular cryo-ET and in-cell NMR offer ideal complementation. FRET detects fluorescence signals from individual molecules in single cells. However, information on cellular localization is limited by diffraction to hundreds of nanometers. Here, cellular cryo-ET measurements at near-atomic resolution are of great complementary value, albeit at the expense of being able to access protein dynamics because samples are frozen or vitrified. Whereas FRET delineates structural and dynamic information based on engineered pairs of fluorophores typically spanning distances of several nanometers on timescales ranging from nanoseconds to hours, NMR can, in principle, report on all protein residues in an appropriately isotope-labeled sample and reveal short-range distance information on local secondary and tertiary structure in a fully residue-resolved manner. In addition, it provides dynamic information on individual backbone and side-chain motions across multiple timescales, as we discuss later. However, owing to its poor sensitivity and general readout modality, it requires averaging over all molecules in the sample. For these reasons, the combined use of FRET and NMR spectroscopy offers excellent mutual benefits. Yet another goal to be accomplished in the future is to harness the impressive developments in optical super-resolution microscopy for in-cell FRET applications. The power of single-molecule FRET measurements in combination with other biophysical techniques has been demonstrated in several recent *in vitro* studies [32,64–67]. We anticipate similar synergistic breakthroughs for joint applications in live cells.

In-cell NMR and EPR

We finally arrive at the subnanometer to atomic resolution level where in-cell nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopy reign [68]. Both methods exploit similar, non-destructive magneto-resonance principles for the detection of biomolecules that are either based on nuclear spins and their selective excitation via radiofrequency pulses (NMR), or on electron spins and their manipulation by microwave irradiation (EPR). Importantly, NMR and EPR are ensemble methods that convey the behavior of molecules in a sample-averaged manner, meaning that respective in-cell NMR or EPR signals originate from many molecules in many cells. Correspondingly, the information content of such samples reflects global rather than individual molecule properties.

Different from in-cell single-molecule FRET measurements, basic in-cell NMR applications do not require chemical modifications or attachment of dyes to proteins to be studied in cells. Instead, proteins are labeled with stable NMR-active isotopes, which enable their selective detection against the backdrop of all intracellular components not containing such isotopes. Specifically, natural abundance protein nitrogen (^{14}N) and carbon (^{12}C) nuclei are replaced with ^{15}N (~0.4% natural abundance) and ^{13}C (~1% natural abundance), respectively, when protein samples are recombinantly produced in bacteria. Upon intracellular delivery, multidimensional (2D, 3D) NMR experiments are then used to correlate and detect ^{15}N or ^{13}C nuclei bound to NMR-active protons (100% natural abundance). Alternatively, ^{1}H–^{15}N and ^{1}H–^{13}C magnetization transfers may be used to further 'connect' ^{15}N–^{13}C, or ^{13}C–^{13}C spin systems. In this manner, isotope-labeled proteins are selectively 'visualized' in cells. Importantly, this mode of detection relies on a quantum mechanical rather than a chemical labeling effect, which ensures that proteins are studied in their native sequence contexts. The higher natural abundance of ^{13}C often reveals signals of endogenous metabolites in 2D ^{1}H–^{13}C in-cell NMR experiments, which can easily be subtracted with reference experiments recorded on cells not containing isotope-labeled protein. Other naturally occurring, NMR-active nuclei such as ^{19}F and ^{31}P (both 100% natural abundance) are either rare (^{19}F), or not bound to protein protons, carbon or nitrogen (^{31}P). Evidently, in-cell FRET and NMR applications require active delivery, or enrichment routines to accumulate fluorescence- or isotope-labeled proteins in target cells. Indeed, much of the recent progress in in-cell NMR spectroscopy relates to the development of such methods [69,70] and of detailed protocols for the recording and interpretation of in-cell NMR spectra [71–74]. Here, we focus on the latest conceptual and methodological advancements and

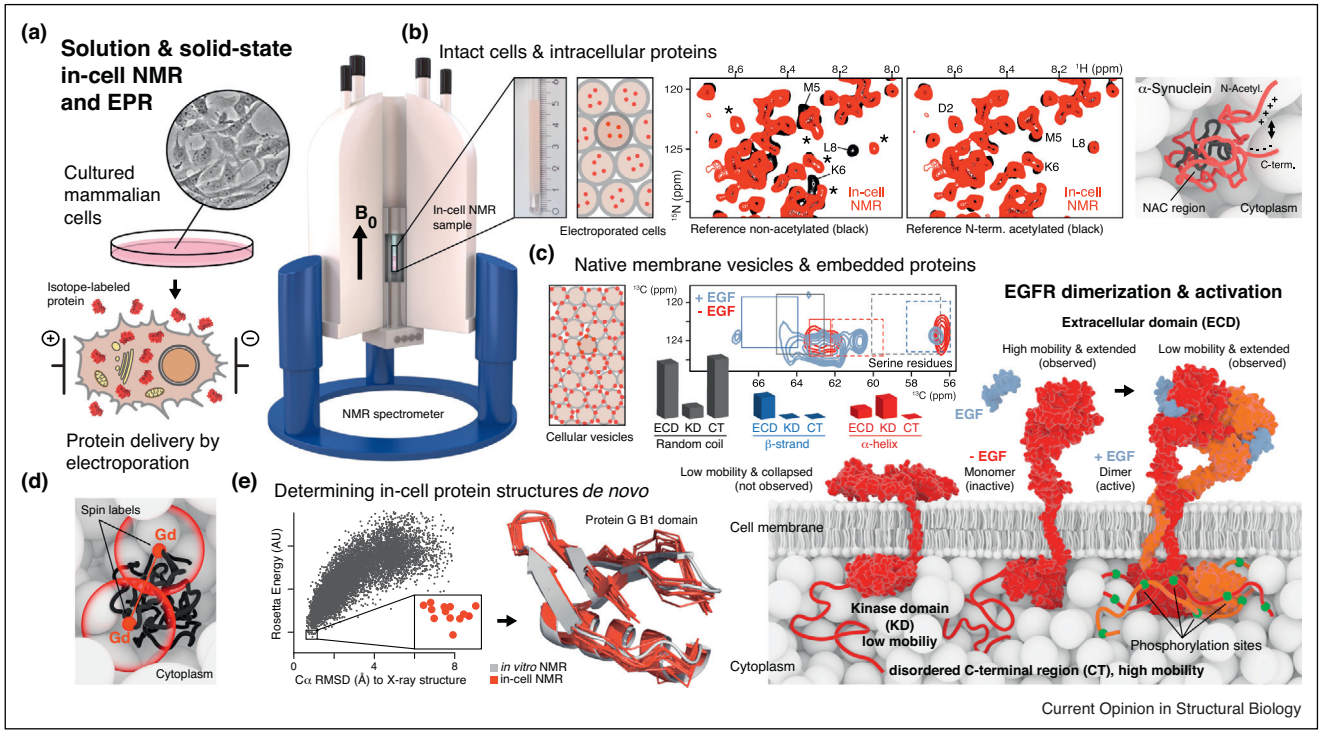
how they interface with in-cell EPR, cryo-ET and single-molecule FRET applications.

On the conceptual side, in-cell NMR experiments proved instrumental in establishing the importance of quinary protein structure as a general phenomenon of folded and disordered proteins in cells ([75] and references therein). Quinary protein structure denotes the fifth level of protein structural organization and describes the combined effects of transient intermolecular interactions acting on proteins in the crowded cytoplasm of prokaryotic and eukaryotic cells. Several studies provided key insights into the nature of these interactions [76] and how they are modulated by general physical properties such as pH and ionic strength [77,78]. They further elucidated quinary structure contributions to intracellular protein stability and folding [76,79] and how they may have evolved in a species-specific manner [80]. On the methodological side, major breakthroughs include the development of a straightforward electroporation protocol to deliver isotope- and differently spin-labeled proteins into cultured mammalian cells, thus making the preparation of mammalian in-cell NMR and EPR samples widely accessible [81,82,83••,84–86] (Figure 4a). Following this approach, Theillet *et al.* delivered the human amyloid protein α-synuclein into five different mammalian cell lines to demonstrate that the disordered state of the non-aggregated, monomeric protein is preserved under physiological cell conditions. They further confirmed that N-terminal acetylation is a constitutive α-synuclein modification in neuronal and non-neuronal mammalian cells (Figure 4b). Importantly, Theillet *et al.* delineated a first comprehensive description of how intracellular viscosity and macromolecular crowding affect the dynamics of individual residues in a non-globular protein. Their results established that fast backbone motions in the millisecond time range remained virtually unchanged, whereas independent electrostatic and hydrophobic quinary structure interactions attenuated motions on the second timescale. Moreover, they found that these interactions were not uniform and mapped to functionally important regions of the protein.

Given the size limitations of solution NMR applications, and the detrimental effects of intracellular viscosity, crowding and quinary structure on signal quality [81,87], in-cell solid-state NMR measurements offer appealing alternatives, especially for studies of rigid macromolecular complexes and membrane proteins in their native environments [69]. Spearheaded by the Baldus group, the structural and dynamic properties of the native membrane-embedded, megadalton type IV secretion system core complex (T4SScc) [73] and the KscA potassium channel [88] were recently studied by in-cell solid-state NMR. Rather than delivering isotope-labeled proteins into cells, these approaches took advantage of selective isotope enrichment by expressing target proteins in cells grown in isotope-containing medium. Common to these applications is the exquisite use of dynamic nuclear polarization (DNP) for signal enhancement, which proved particularly powerful in efforts to explore the structure and dynamics of epidermal growth factor receptor (EGFR) activation upon EGF stimulation [89••] (Figure 4c). Specifically, Kaplan *et al.* employed native membrane vesicles of human A431 cells expressing endogenous levels of EFGR to establish that EGF ligand binding restricts intra- and extra-cellular receptor domain motions. In turn, they speculated that this reduction in conformational entropy provides the basis for EGFR dimerization and activation. Elegant experiments on prion fibrils of the Sup35 protein in yeast- [90] and of Bcl-X_L in *E. coli* cell-lysates [91•] further emphasized the power of such DNP-based solid-state NMR approaches. The comparatively large sizes of these biomolecular assemblies transcend into the realm of possible cryo-ET applications. One particularly interesting area for such complementary studies could be the analysis of native amyloid fibrils formed directly in cultured human cells, or in patient-derived cellular preparations [92,93]. While non-aggregated, 'healthy' protein states could be investigated by in-cell single-molecule FRET and solution NMR methods, oligomers, fibrils and other higher-order aggregates could be targeted by solid-state in-cell NMR and cryo-ET experiments. The recent establishment of in-cell EPR spectroscopy, which is not limited by protein size, may offer valuable additional insights in such collaborative efforts.

In contrast to NMR, EPR measurements necessitate site-directed spin labeling of target proteins at chosen positions, similar to FRET applications. EPR labels are often based on organic compounds with unpaired electrons or, as preferred for in-cell applications, on macrocyclic chelators such as DOTA and stably coordinated paramagnetic lanthanide metals [83••,86,94]. Thus, in-cell FRET and EPR applications may suffer from similar shortcomings regarding the influence of tags on the structural and dynamic properties of delivered proteins. These drawbacks are offset by the opportunity to generate FRET and EPR samples from the same starting material, typically engineered protein cysteine variants for fluorophore- or spin-label coupling, and the ability to directly compare in-cell FRET and EPR results. Contrary to the need for orthogonal dye pairs at two protein positions for FRET measurements, identical spin labels may be added at two sites for spin–spin distance measurements by double electron–electron resonance (DEER) experiments [95] (Figure 4d). Thus, in-cell EPR-DEER and in-cell FRET measurements employ congruent rationales and provide complementary results; spin–spin distances in the case of EPR-DEER, fluorophore–fluorophore distances in the case of FRET. Whereas FRET experiments yield single-molecule, single-cell distance information that is locally and temporally resolved, EPR-DEER results are ensemble-averaged over many molecules in many

Figure 4



In-cell NMR and EPR spectroscopy. **(a)** Schematic overview of the delivery of isotope-labeled proteins into cultured mammalian cells by electroporation for preparing solution in-cell NMR and EPR samples. **(b)** Intact electroporated cells are directly transferred into NMR tubes and sedimented for measurements on the resulting cell slurries. NMR spectra depict results from 2D ^{1}H–^{15}N correlation experiments on the human amyloid protein α-synuclein in isolation (black) and in A2780 cells (red). Non-acetylated protein is shown in the reference spectrum on the left, N-terminally acetylated reference α-synuclein is shown on the right. Non-matching in-cell NMR cross-peaks of N-terminal α-synuclein resonances in the left panel are marked with asterisks. Their perfect superposition with NMR signals of N-terminally acetylated protein is evident on the right. Model representation of cellular α-synuclein (red) shielding its amyloidogenic NAC region (black) from interactions with cytoplasmic components shown schematically as white spheres. Transient electrostatic interactions between N- and C-terminal residues are indicated (adapted from Theillet *et al.* [83••]). **(c)** Overview of solid-state NMR experiments on cellular vesicles prepared from native membranes of A431 cells expressing endogenous amounts of epidermal growth-factor receptor (EGFR). The overlay of 2D NMR spectra show ^{13}C–^{13}C correlations of EGFR serine residues in the absence (red) and presence (blue) of EGF growth-factor stimulation, with carbon chemical-shift values indicative of random coil, β-strand and α-helical conformations boxed in gray, blue and red, respectively. Bar graphs outline the distribution of secondary structure elements within the extracellular domain (ECD), kinase domain (KD) and C-terminal region (CT) of cellular EGFR as determined by *in situ* NMR measurements (adapted from Kaplan *et al.* [89••]). Models from left to right depict possible EGFR conformations upon activation by EGF (blue), according to NMR data (bottom-right panel). In the absence of growth factor, the ECD of native monomeric EGFR exists in an extended, high-mobility conformation rather than in a membrane-collapsed low-mobility state previously discussed as a possible conformation of inactive EGFR. Upon EGF binding and receptor dimerization, ECD residues display reduced mobility (far right). In this state, cross-phosphorylation of EGFR CT residues (shown as green balls) by the cytoplasmic kinase domains initiates downstream signaling. Models were prepared with coordinates provided in Arkhipov *et al.* [102]. **(d)** Schematic representation of double electron–electron resonance (DEER) coupling of Gadolinium (Gd) spin-labeled α-synuclein in mammalian cells. **(e)** In-cell protein structures and energies calculated with Rosetta using pseudocontact shift (PCS) and residual dipolar coupling (RDC) 2D in-cell NMR data of the protein G B1 domain (GB1) as input. Ribbon presentations depict the superposition of 13 lowest-energy Rosetta structures (red) with the *in vitro* NMR structure of GB1 (gray, PDB ID: 1GB1, adapted from Müntener *et al.* [97]).

cells without spatial resolution. Clearly, the combined use of both methods provides the most comprehensive insights.

Further extending the complementarity of in-cell NMR and EPR applications, lanthanide spin labels can also be used for paramagnetic relaxation enhancement (PRE) measurements by NMR spectroscopy, either to derive long-range distance information about intracellular protein conformations [83••], or to induce pseudocontact shifts (PCSs) and residual dipolar couplings (RDCs) to determine entire protein structures from simple 2D NMR experiments [85,96,97] (Figure 4e). Alternative in-cell structure determination routines in bacteria, based on classical 3D NMR methods [98] were recently advanced for samples at closer-to-physiological protein concentrations [71]. Together with the aforementioned 2D NMR approaches, they constitute a sophisticated toolkit for solving protein structures in prokaryotic and eukaryotic cells *de novo*.

While folded proteins will represent the major focus of future solid-state in-cell NMR projects, structural studies of disordered proteins and protein regions are likely to dominate applications in solution. Given the advantageous dynamic and relaxation properties of these proteins *in vitro* and in cells, solution NMR is particularly well poised to interrogate their intracellular dynamics and conformational ensemble properties [99]. Both solution and solid-state NMR methods will continue to thrive in their unique ability to provide quantitative insights into different types of protein motions spanning timescales from picoseconds to hours.

Outlook

A consolidated view of suitable methods for future applications in Cellular Structural Biology is emerging. Above all, the integrated use of complementary single- and ensemble-molecule methods operating at different levels of resolution presents itself as the most promising route for comprehensive in-cell studies in the years to come. The impact of such studies will depend on strategic alliances and concerted actions to harness the powers of these tools in the best possible ways. While this can be achieved through individual collaborations, it will benefit from broader, community-wide efforts including dedicated training programs at the interface of cell biology and biophysics. Biological aspects in particular need to be strengthened, especially with regard to measurements in physiologically relevant cell types. Many of the current *in situ* approaches exploit generic, immortalized laboratory cell lines that are robust and easy to handle. However, they may fall short of representing the most suitable biological context for the question under investigation. Here, primary cells or dissected tissue sections provide unique advantages, especially for methods that do not require large sample cell numbers, that is, cryo-ET and single-molecule FRET. Whenever high cell numbers are needed, as is the case for in-cell NMR and EPR experiments, the use of induced pluripotent stem cells (iPSCs) and their differentiation into specialized cellular lineages offers appealing alternatives. Such considerations underscore the need for advanced expertise in different areas of cell biology, which is, ideally, jointly available with technical and practical know-how of sophisticated biophysical methods. Future in-cell studies will further benefit from newly established *in situ* methods such as cross-linking mass spectrometry [100], correlative light and electron microscopy (CLEM) and super-resolution fluorescence microscopy [101], which already play important roles in cryo-ET applications [27–29]. Their use in in-cell NMR, EPR and single-molecule FRET studies will produce equally desirable synergies.

To accomplish these goals, we must also learn to embrace the conceptual void that separates the precise nature of Structural Biology methods from the complexity and poor quantitative definition of biological systems. For the time being, we may have to accept the notion that measurements in cellular environments are inherently 'noisy' and bound to produce bias and uncertainties. We shall face these drawbacks with vigor and strive to overcome them, especially in light of the overarching cause: To comprehensively understand biology in all of its beauty and complex detail.

Conflict of interest

None declared.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Ito Y, Selenko P: **Cellular structural biology**. *Curr Opin Struct Biol* 2010, **20**:640-648.

2. Irobalieva RN, Martins B, Medalia O: **Cellular structural biology as revealed by cryo-electron tomography**. *J Cell Sci* 2016, **129**:469-476.

3. Theillet FX, 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.

4. McMullan G, Faruqi AR, Henderson R: **Direct electron detectors**. *Methods Enzymol* 2016, **579**:1-17.

5. Ripstein ZA, Rubinstein JL: **Processing of cryo-EM movie data**. *Methods Enzymol* 2016, **579**:103-124.

6. Passmore LA, Russo CJ: **Specimen preparation for high-resolution cryo-EM**. *Methods Enzymol* 2016, **579**:51-86.

7. Bai XC, McMullan G, Scheres SH: **How cryo-EM is revolutionizing structural biology**. *Trends Biochem Sci* 2015, **40**:49-57.

8. Orlov I, Myasnikov AG, Andronov L, Natchiar SK, Khatter H, Beinsteiner B, Menetret JF, Hazemann I, Mohideen K, Tazibt K *et al.*: **The integrative role of cryo electron microscopy in molecular and cellular structural biology**. *Biol Cell* 2017, **109**:81-93.

9. • Beck M, Baumeister W: **Cryo-electron tomography: can it reveal the molecular sociology of cells in atomic detail?** *Trends Cell Biol* 2016, **26**:825-837.
An excellent review about the current state of cryo-electron tomography. The authors focus on its application for *in situ* structural biology and clearly describe the principles, limitations and prospects of the method.

10. • Wan W, Briggs JA: **Cryo-electron tomography and subtomogram averaging**. *Methods Enzymol* 2016, **579**:329-367.
This and other contributions in the 579 Volume of Methods in Enzymology provide comprehensive descriptions of state of the art technologies in cryo-electron microscopy. Each chapter covers different methodological aspects and individual areas of application.

11. Rigort A, Bauerlein FJ, Villa E, Eibauer M, Laugks T, Baumeister W, Plitzko JM: **Focused ion beam micromachining of eukaryotic**

cells for cryoelectron tomography. *Proc Natl Acad Sci U S A* 2012, **109**:4449-4454.

12. Schaffer M, Mahamid J, Engel BD, Laugks T, Baumeister W, Plitzko JM: **Optimized cryo-focused ion beam sample preparation aimed at in situ structural studies of membrane proteins**. *J Struct Biol* 2017, **197**:73-82.

13. Villa E, Schaffer M, Plitzko JM, Baumeister W: **Opening windows into the cell: focused-ion-beam milling for cryo-electron tomography**. *Curr Opin Struct Biol* 2013, **23**:771-777.

14. Danev R, Buijsse B, Khoshouei M, Plitzko JM, Baumeister W: **Volta potential phase plate for in-focus phase contrast transmission electron microscopy**. *Proc Natl Acad Sci U S A* 2014, **111**:15635-15640.

15. Khoshouei M, Pfeffer S, Baumeister W, Forster F, Danev R: **Subtomogram analysis using the Volta phase plate**. *J Struct Biol* 2017, **197**:94-101.

16. Fukuda Y, Laugks U, Lucic V, Baumeister W, Danev R: **Electron cryotomography of vitrified cells with a Volta phase plate**. *J Struct Biol* 2015, **190**:143-154.

17. Engel BD, Schaffer M, Kuhn Cuellar L, Villa E, Plitzko JM, Baumeister W: **Native architecture of the *Chlamydomonas* chloroplast revealed by in situ cryo-electron tomography**. *Elife* 2015, **4**.

18. Engel BD, Schaffer M, Albert S, Asano S, Plitzko JM, Baumeister W: **In situ structural analysis of Golgi intracisternal protein arrays**. *Proc Natl Acad Sci U S A* 2015, **112**:11264-11269.

19. Asano S, Fukuda Y, Beck F, Aufderheide A, Forster F, Danev R, Baumeister W: **Proteasomes. A molecular census of 26S proteasomes in intact neurons**. *Science* 2015, **347**:439-442.

20. Harapin J, Bormel M, Sapra KT, Brunner D, Kaech A, Medalia O: **Structural analysis of multicellular organisms with cryo-electron tomography**. *Nat Methods* 2015, **12**:634-636.

21. Mahamid J, Schampers R, Persoon H, Hyman AA, Baumeister W, Plitzko JM: **A focused ion beam milling and lift-out approach for site-specific preparation of frozen-hydrated lamellas from multicellular organisms**. *J Struct Biol* 2015, **192**:262-269.

22. Pfeffer S, Dudek J, Schaffer M, Ng BG, Albert S, Plitzko JM, Baumeister W, Zimmermann R, Freeze HH, Engel BD *et al.*: **Dissecting the molecular organization of the translocon-associated protein complex**. *Nat Commun* 2017, **8**:14516.

23. Stoffler D, Feja B, Fahrenkrog B, Walz J, Typke D, Aebi U: **Cryo-electron tomography provides novel insights into nuclear pore architecture: implications for nucleocytoplasmic transport**. *J Mol Biol* 2003, **328**:119-130.

24. Beck M, Forster F, Ecke M, Plitzko JM, Melchior F, Gerisch G, Baumeister W, Medalia O: **Nuclear pore complex structure and dynamics revealed by cryoelectron tomography**. *Science* 2004, **306**:1387-1390.

25. Beck M, Lucic V, Forster F, Baumeister W, Medalia O: **Snapshots of nuclear pore complexes in action captured by cryo-electron tomography**. *Nature* 2007, **449**:611-615.

26. Eibauer M, Pellanda M, Turgay Y, Dubrovsky A, Wild A, Medalia O: **Structure and gating of the nuclear pore complex**. *Nat Commun* 2015, **6**:7532.

27. Bui KH, von Appen A, DiGuilio AL, Ori A, Sparks L, Mackmull MT, Bock T, Hagen W, Andres-Pons A, Glavy JS *et al.*: **Integrated structural analysis of the human nuclear pore complex scaffold**. *Cell* 2013, **155**:1233-1243.

28. von Appen A, Kosinski J, Sparks L, Ori A, DiGuilio AL, Vollmer B, Mackmull MT, Banterle N, Parca L, Kastritis P *et al.*: **In situ structural analysis of the human nuclear pore complex**. *Nature* 2015, **526**:140-143.

29. Kosinski J, Mosalaganti S, von Appen A, Teimer R, DiGuilio AL, Wan W, Bui KH, Hagen WJ, Briggs JA, Glavy JS *et al.*: **Molecular architecture of the inner ring scaffold of the human nuclear pore complex**. *Science* 2016, **352**:363-365.

30. Maimon T, Elad N, Dahan I, Medalia O: **The human nuclear pore complex as revealed by cryo-electron tomography**. *Structure* 2012, **20**:998-1006.

31. •• Mahamid J, Pfeffer S, Schaffer M, Villa E, Danev R, Cuellar LK, Forster F, Hyman AA, Plitzko JM, Baumeister W: **Visualizing the molecular sociology at the HeLa cell nuclear periphery**. *Science* 2016, **351**:969-972.
Cryo-FIB milling, direct electron detection and Volta phase plates are jointly used to identify molecular components based on their structural signatures alone, setting the stage for 'visual proteomics'.

32. Milles S, Mercadante D, Aramburu IV, Jensen MR, Banterle N, Koehler C, Tyagi S, Clarke J, Shammas SL, Blackledge M *et al.*: **Plasticity of an ultrafast interaction between nucleoporins and nuclear transport receptors**. *Cell* 2015, **163**:734-745.

33. Ader C, Frey S, Maas W, Schmidt HB, Gorlich D, Baldus M: **Amyloid-like interactions within nucleoporin FG hydrogels**. *Proc Natl Acad Sci U S A* 2010, **107**:6281-6285.

34. Hough LE, Dutta K, Sparks S, Temel DB, Kamal A, Tetenbaum-Novatt J, Rout MP, Cowburn D: **The molecular mechanism of nuclear transport revealed by atomic-scale measurements**. *Elife* 2015, **4**.

35. Joo C, Balci H, Ishitsuka Y, Buranachai C, Ha T: **Advances in single-molecule fluorescence methods for molecular biology**. *Annu Rev Biochem* 2008, **77**:51-76.

36. Schuler B, Hofmann H: **Single-molecule spectroscopy of protein folding dynamics-expanding scope and timescales**. *Curr Opin Struct Biol* 2013, **23**:36-47.

37. Dunkle JA, Cate JH: **Ribosome structure and dynamics during translocation and termination**. *Annu Rev Biophys* 2010, **39**:227-244.

38. Marshall RA, Aitken CE, Dorywalska M, Puglisi JD: **Translation at the single-molecule level**. *Annu Rev Biochem* 2008, **77**:177-203.

39. Kapanidis AN, Strick T: **Biology, one molecule at a time**. *Trends Biochem Sci* 2009, **34**:234-243.

40. Ha T, Kozlov AG, Lohman TM: **Single-molecule views of protein movement on single-stranded DNA**. *Annu Rev Biophys* 2012, **41**:295-319.

41. Smiley RD, Hammes GG: **Single molecule studies of enzyme mechanisms**. *Chem Rev* 2006, **106**:3080-3094.

42. Blank K, De Cremer G, Hofkens J: **Fluorescence-based analysis of enzymes at the single-molecule level**. *Biotechnol J* 2009, **4**:465-479.

43. Aleman EA, Lamichhane R, Rueda D: **Exploring RNA folding one molecule at a time**. *Curr Opin Chem Biol* 2008, **12**:647-654.

44. Muschielok A, Andrecka J, Jawhari A, Bruckner F, Cramer P, Michaelis J: **A nano-positioning system for macromolecular structural analysis**. *Nat Methods* 2008, **5**:965-971.

45. Kalinin S, Peulen T, Sindbert S, Rothwell PJ, Berger S, Restle T, Goody RS, Gohlke H, Seidel CA: **A toolkit and benchmark study for FRET-restrained high-precision structural modeling**. *Nat Methods* 2012, **9**:1218-1225.

46. Hellenkamp B, Wortmann P, Kandzia F, Zacharias M, Hugel T: **Multidomain structure and correlated dynamics determined by self-consistent FRET networks**. *Nat Methods* 2017, **14**:174-180.

47. Dimura M, Peulen TO, Hanke CA, Prakash A, Gohlke H, Seidel CA: **Quantitative FRET studies and integrative modeling unravel the structure and dynamics of biomolecular systems**. *Curr Opin Struct Biol* 2016, **40**:163-185.

48. Tsien RY: **Constructing and exploiting the fluorescent protein paintbox (Nobel Lecture)**. *Angew Chem* 2009, **48**:5612-5626.

49. Greife A, Felekyan S, Ma Q, Gertzen CG, Spomer L, Dimura M, Peulen TO, Wohler C, Haussinger D, Gohlke H *et al.*: **Structural assemblies of the di- and oligomeric G-protein coupled receptor TGR5 in live cells: an MFIS-FRET and integrative modelling study**. *Sci Rep* 2016, **6**:36792.

50. • Kravets E, Degrandi D, Ma Q, Peulen TO, Klumpers V, Felekyan S, Kuhnemuth R, Weidtkamp-Peters S, Seidel CA, Pfeffer K: **Guanylate binding proteins directly attack *Toxoplasma gondii* via supramolecular complexes**. *Elife* 2016, **5**.
This work illustrates how ensemble-averaged detection using multi-parameter fluorescence image spectroscopy can be used to validate structural models and determine equilibrium constants in live cells.

51. Gnutt D, Ebbinghaus S: **The macromolecular crowding effect — from in vitro into the cell**. *Biol Chem* 2016, **397**:37-44.

52. Boersma AJ, Zuhorn IS, Poolman B: **A sensor for quantification of macromolecular crowding in living cells**. *Nat Methods* 2015, **12**:227-229.

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

54. Dhar A, Girdhar K, Singh D, Gelman H, Ebbinghaus S, Gruebele M: **Protein stability and folding kinetics in the nucleus and endoplasmic reticulum of eucaryotic cells**. *Biophys J* 2011, **101**:421-430.

55. Murakoshi H, Iino R, Kobayashi T, Fujiwara T, Ohshima C, Yoshimura A, Kusumi A: **Single-molecule imaging analysis of Ras activation in living cells**. *Proc Natl Acad Sci U S A* 2004, **101**:7317-7322.

56. •• König I, Zarrine-Afsar A, Aznauryan M, Soranno A, Wunderlich B, Dingfelder F, Stuber JC, Pluckthun A, Nettels D, Schuler B: **Single-molecule spectroscopy of protein conformational dynamics in live eukaryotic cells**. *Nat Methods* 2015, **12**:773-779.
Extension of single-molecule FRET in live eukaryotic cells to the investigation of protein dynamics, conformational stability and folding kinetics.

57. Sustarsic M, Kapanidis AN: **Taking the ruler to the jungle: single-molecule FRET for understanding biomolecular structure and dynamics in live cells**. *Curr Opin Struct Biol* 2015, **34**:52-59.

58. •• Sakon JJ, Weninger KR: **Detecting the conformation of individual proteins in live cells**. *Nat Methods* 2010, **7**:203-205.
The first application of single-molecule FRET to detect conformational changes in live eukaryotic cells, based on total internal reflection microscopy and microinjection.

59. Hoffmann A, Nettels D, Clark J, Borgia A, Radford SE, Clarke J, Schuler B: **Quantifying heterogeneity and conformational dynamics from single molecule FRET of diffusing molecules: recurrence analysis of single particles (RASP)**. *Phys Chem Chem Phys* 2011, **13**:1857-1871.

60. Fessl T, Adamec F, Polivka T, Foldynova-Trantirkova S, Vacha F, Trantirek L: **Towards characterization of DNA structure under physiological conditions in vivo at the single-molecule level using single-pair FRET**. *Nucleic Acids Res* 2012, **40**:e121.

61. •• Crawford R, Torella JP, Aigrain L, Plochowietz A, Gryte K, Uphoff S, Kapanidis AN: **Long-lived intracellular single-molecule fluorescence using electroporated molecules**. *Biophys J* 2013, **105**:2439-2450.
The authors establish the use of electroporation for delivering fluorescently labeled protein into bacterial cells and monitoring single-molecule FRET.

62. Stracy M, Uphoff S, Garza de Leon F, Kapanidis AN: **In vivo single-molecule imaging of bacterial DNA replication, transcription, and repair**. *FEBS Lett* 2014, **588**:3585-3594.

63. Chen H, Farkas ER, Webb WW: **Chapter 1: In vivo applications of fluorescence correlation spectroscopy**. *Methods Cell Biol* 2008, **89**:3-35.

64. Campos LA, Sadqi M, Liu J, Wang X, English DS, Munoz V: **Gradual disordering of the native state on a slow two-state folding protein monitored by single-molecule fluorescence spectroscopy and NMR**. *J Phys Chem B* 2013, **117**:13120-13131.

65. Aznauryan M, Delgado L, Soranno A, Nettels D, Huang JR, Labhardt AM, Grzesiek S, Schuler B: **Comprehensive structural and dynamical view of an unfolded protein from the combination of single-molecule FRET, NMR, and SAXS**. *Proc Natl Acad Sci U S A* 2016, **113**:E5389-E5398.

66. Borgia A, Zheng W, Buholzer K, Borgia MB, Schuler A, Hofmann H, Soranno A, Nettels D, Gast K, Grishaev A *et al.*: **Consistent view of polypeptide chain expansion in chemical denaturants from multiple experimental methods**. *J Am Chem Soc* 2016, **138**:11714-11726.

67. Boura E, Rozycki B, Herrick DZ, Chung HS, Vecer J, Eaton WA, Cafiso DS, Hummer G, Hurley JH: **Solution structure of the ESCRT-I complex by small-angle X-ray scattering, EPR, and FRET spectroscopy**. *Proc Natl Acad Sci U S A* 2011, **108**:9437-9442.

68. Freedberg DI, Selenko P: **Live cell NMR**. *Annu Rev Biophys* 2014, **43**:171-192.

69. Warnet XL, Arnold AA, Marcotte I, Warschawski DE: **In-cell solid-state NMR: an emerging technique for the study of biological membranes**. *Biophys J* 2015, **109**:2461-2466.

70. Luchinat E, Banci L: **A unique tool for cellular structural biology: in-cell NMR**. *J Biol Chem* 2016, **291**:3776-3784.

71. Ikeya T, Hanashima T, Hosoya S, Shimazaki M, Ikeda S, Mishima M, Guntert P, Ito Y: **Improved in-cell structure determination of proteins at near-physiological concentration**. *Sci Rep* 2016, **6**:38312.

72. Felli IC, Gonnelli L, Pierattelli R: **In-cell (1)(3)C NMR spectroscopy for the study of intrinsically disordered proteins**. *Nat Protoc* 2014, **9**:2005-2016.

73. Kaplan M, Cukkemane A, van Zundert GC, Narasimhan S, Daniels M, Mance D, Waksman G, Bonvin AM, Fronzes R, Folkers GE *et al.*: **Probing a cell-embedded megadalton protein complex by DNP-supported solid-state NMR**. *Nat Methods* 2015, **12**:649-652.

74. Barbieri L, Luchinat E, Banci L: **Characterization of proteins by in-cell NMR spectroscopy in cultured mammalian cells**. *Nat Protoc* 2016, **11**:1101-1111.

75. 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.

76. Smith AE, Zhou LZ, Gorensek AH, Senske M, Pielak GJ: **In-cell thermodynamics and a new role for protein surfaces**. *Proc Natl Acad Sci U S A* 2016, **113**:1725-1730.

77. Cohen RD, Guseman AJ, Pielak GJ: **Intracellular pH modulates quinary structure**. *Protein Sci* 2015, **24**:1748-1755.

78. Cohen RD, Pielak GJ: **Electrostatic contributions to protein quinary structure**. *J Am Chem Soc* 2016 http://dx.doi.org/10.1021/jacs.6b07323.

79. Monteith WB, Cohen RD, Smith AE, Guzman-Cisneros E, Pielak GJ: **Quinary structure modulates protein stability in cells**. *Proc Natl Acad Sci U S A* 2015, **112**:1739-1742.

80. Zhang J, Yang JR: **Determinants of the rate of protein sequence evolution**. *Nat Rev Genet* 2015, **16**:409-420.

81. Majumder S, Xue J, DeMott CM, Reverdatto S, Burz DS, Shekhtman A: **Probing protein quinary interactions by in-cell nuclear magnetic resonance spectroscopy**. *Biochemistry* 2015, **54**:2727-2738.

82. Danielsson J, Mu X, Lang L, Wang H, Binolfi A, Theillet FX, Bekei B, Logan DT, Selenko P, Wennerstrom H *et al.*: **Thermodynamics of protein destabilization in live cells**. *Proc Natl Acad Sci U S A* 2015, **112**:12402-12407.

83. •• Theillet FX, Binolfi A, Bekei B, Martorana A, Rose HM, Stuiver M, Verzini S, Lorenz D, van Rossum M, Goldfarb D *et al.*: **Structural disorder of monomeric alpha-synuclein persists in mammalian cells**. *Nature* 2016, **530**:45-50.
First full-scale in-cell NMR and EPR analysis of the structure and dynamics of a human amyloid protein in neuronal and non-neuronal cell types, introducing protein electroporation as a tool to deliver isotope-labeled and spin-labeled protein into cultured mammalian cells.

84. Binolfi A, Limatola A, Verzini S, Kosten J, Theillet FX, Rose HM, Bekei B, Stuiver M, van Rossum M, Selenko P: **Intracellular repair of oxidation-damaged alpha-synuclein fails to target C-terminal modification sites**. *Nat Commun* 2016, **7**:10251.

85. Hikone Y, Hirai G, Mishima M, Inomata K, Ikeya T, Arai S, Shirakawa M, Sodeoka M, Ito Y: **A new carbamidemethyl-linked**

lanthanoid chelating tag for PCS NMR spectroscopy of proteins in living HeLa cells. *J Biomol NMR* 2016, **66**:99-110.

86. Yang Y, Yang F, Gong YJ, Chen JL, Goldfarb D, Su XC: **A reactive, rigid GdIII labeling tag for in-cell EPR distance measurements in proteins**. *Angew Chem Int Ed Engl* 2017, **56**:2914-2918.

87. Barbieri L, Luchinat E, Banci L: **Protein interaction patterns in different cellular environments are revealed by in-cell NMR**. *Sci Rep* 2015, **5**:14456.

88. Medeiros-Silva J, Mance D, Daniels M, Jekhmane S, Houben K, Baldus M, Weingarth M: **^{1}H-detected solid-state nmr studies of water-inaccessible proteins in vitro and in situ**. *Angew Chem Int Ed Engl* 2016, **55**:13606-13610.

89. •• Kaplan M, Narasimhan S, de Heus C, Mance D, van Doorn S, Houben K, Popov-Celeketic D, Damman R, Katrukha EA, Jain P *et al.*: **EGFR dynamics change during activation in native membranes as revealed by NMR**. *Cell* 2016, **167** 1241–1251.e11.
First in-cell solid-state NMR study of a selectively isotope-labeled, endogenous membrane protein in native vesicles of cultured human cells, exploiting dynamic nuclear polarization for signal enhancement.

90. Frederick KK, Michaelis VK, Corzilius B, Ong TC, Jacavone AC, Griffin RG, Lindquist S: **Sensitivity-enhanced NMR reveals alterations in protein structure by cellular milieus**. *Cell* 2015, **163**:620-628.

91. • Viennet T, Viegas A, Kuepper A, Arens S, Gelev V, Petrov O, Grossmann TN, Heise H, Etzkorn M: **Selective protein hyperpolarization in cell lysates using targeted dynamic nuclear polarization**. *Angew Chem Int Ed Engl* 2016, **55**:10746-10750.
Elegant demonstration of ligand-induced targeting of hyperpolarization effects for the enhancement of protein solid-state NMR signals in native bacterial cell lysates.

92. Lu JX, Qiang W, Yau WM, Schwieters CD, Meredith SC, Tycko R: **Molecular structure of beta-amyloid fibrils in Alzheimer's disease brain tissue**. *Cell* 2013, **154**:1257-1268.

93. Qiang W, Yau WM, Lu JX, Collinge J, Tycko R: **Structural variation in amyloid-beta fibrils from Alzheimer's disease clinical subtypes**. *Nature* 2017, **541**:217-221.

94. Martorana A, Bellapadrona G, Feintuch A, Di Gregorio E, Aime S, Goldfarb D: **Probing protein conformation in cells by EPR distance measurements using Gd^{3+} spin labeling**. *J Am Chem Soc* 2014, **136**:13458-13465.

95. Goldfarb D: **Gd^{3+} spin labeling for distance measurements by pulse EPR spectroscopy**. *Phys Chem Chem Phys* 2014, **16**:9685-9699.

96. Pan BB, Yang F, Ye Y, Wu Q, Li C, Huber T, Su XC: **3D structure determination of a protein in living cells using paramagnetic NMR spectroscopy**. *Chem Commun (Camb)* 2016, **52**:10237-10240.

97. Müntener T, Haussinger D, Selenko P, Theillet FX: **In-cell protein structures from 2D NMR experiments**. *J Phys Chem Lett* 2016, **7**:2821-2825.

98. Sakakibara D, Sasaki A, Ikeya T, Hamatsu J, Hanashima T, Mishima M, Yoshimasu M, Hayashi N, Mikawa T, Walchli M *et al.*: **Protein structure determination in living cells by in-cell NMR spectroscopy**. *Nature* 2009, **458**:102-105.

99. Sormanni P, Piovesan D, Heller GT, Bonomi M, Kukic P, Camilloni C, Fuxreiter M, Dosztanyi Z, Pappu RV, Babu MM *et al.*: **Simultaneous quantification of protein order and disorder**. *Nat Chem Biol* 2017, **13**:339-342.

100. Lossl P, van de Waterbeemd M, Heck AJ: **The diverse and expanding role of mass spectrometry in structural and molecular biology**. *EMBO J* 2016, **35**:2634-2657.

101. Wolff G, Hagen C, Grunewald K, Kaufmann R: **Towards correlative super-resolution fluorescence and electron cryo-microscopy**. *Biol Cell* 2016, **108**:245-258.

102. Arkhipov A, Shan Y, Das R, Endres NF, Eastwood MP, Wemmer DE, Kuriyan J, Shaw DE: **Architecture and membrane interactions of the EGF receptor**. *Cell* 2013, **152**:557-569.