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Cellular structural biology
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While we appreciate the complexity of the intracellular environment as a general property of every living organism, we collectively lack the appropriate tools to analyze protein structures in a cellular context. In-cell NMR spectroscopy represents a novel biophysical tool to investigate the conformational and functional characteristics of biomolecules at the atomic level inside live cells. Here, we review recent in-cell NMR developments and provide an outlook towards future applications in prokaryotic and eukaryotic cells. We hope to thereby emphasize the usefulness of in-cell NMR techniques for cellular studies of complex biological processes and for structural analyses in native environments.

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Introduction
The past decade has seen tremendous advances in high-resolution cellular imaging techniques [1]. Recent developments in the field of super-resolution light microscopy have propelled our understanding of many biological processes and provided a wealth of novel spatiotemporal insights into basic cellular activities [2]. Cryo-electron tomography has likewise stunned the scientific community with seemingly complete maps of the cellular interior, and at a yet unknown degree of 'morphological' resolution [3,4] (Figure 1a). With those experimental images at hand, we are beginning to appreciate the true extent of cellular macromolecular crowding [5,6] and to grasp the overwhelming complexity of the intracellular environment (Figure 1b). While both super-resolution microscopy and electron tomography will continue to provide major insights into the inner state of the cell, neither method can answer questions about the fine

details of protein structures *in vivo*. This is why we believe that continuous efforts in developing novel high-resolution *in vivo* methods are urgently needed. Unfortunately, most of the candidate techniques like X-ray crystallography, electron microscopy and solid-state NMR spectroscopy, are unsuitable for *in vivo* applications due to their requirements for pure samples, prohibitively large sample concentrations, and crystalline or vitrified specimens. Liquid-state NMR spectroscopy, by contrast, allows for direct observations of NMR-active nuclei within any NMR-inactive environment and can thus also be employed to investigate the structures of appropriately-labeled biomolecules inside live cells [7].

In-cell NMR spectroscopy represents a cunning extension of a basic magnetic resonance (MR) principle: Most atomic nuclei in natural substances, with the exception of protons (^{1}H), are NMR-insensitive and hence not detected by NMR methods. Therefore, these nuclei are substituted with stable isotopes in order to make them NMR 'visible'. Today, isotope labeling constitutes a routine procedure in biomolecular NMR spectroscopy. Besides labeling all residues of a protein, more sophisticated schemes have been devised to introduce NMR-active isotopes at specific protein positions (i.e. segmental labeling [8•], site selective labeling [9•]), or at subsets of amino acid residues (i.e. residue-specific labeling [10•]). Together, these techniques allow us to either visualize whole proteins, or to engineer NMR observables at specific protein sites. For in-cell NMR applications, the isotope effect is exploited in a selective, filter-like manner. By analyzing isotope-labeled proteins in complex but NMR-inactive mixtures, that is, intracellular environments, in-cell NMR directly assesses the conformational and functional properties of proteins in a cellular context. High-resolution in-cell NMR spectroscopy is not an imaging technique and must therefore not be confused with methods in magneto resonance imaging (MRI). In-cell NMR, like high-resolution *in vitro* NMR, reports the resonance states of coupled spin systems of atomic nuclei. Such resonances are typically influenced by many factors, including the spatial orientation of one atomic nucleus in a protein with respect to another (i.e. the conformation of the structure in which the atoms reside). Therefore, in-cell NMR spectroscopy does not produce an image of the cellular state of a protein, but conveys structural details in an indirect manner. It is important to emphasize that in-cell NMR spectroscopy is not an *in vivo* method in the 'true' biological sense. In-cell NMR always relies on some form of sample delivery that introduces NMR-observable biomolecules into the unlabeled cellular environment. By introducing these observables, in-cell NMR measurements off-set the native cellular state and

Figure 1

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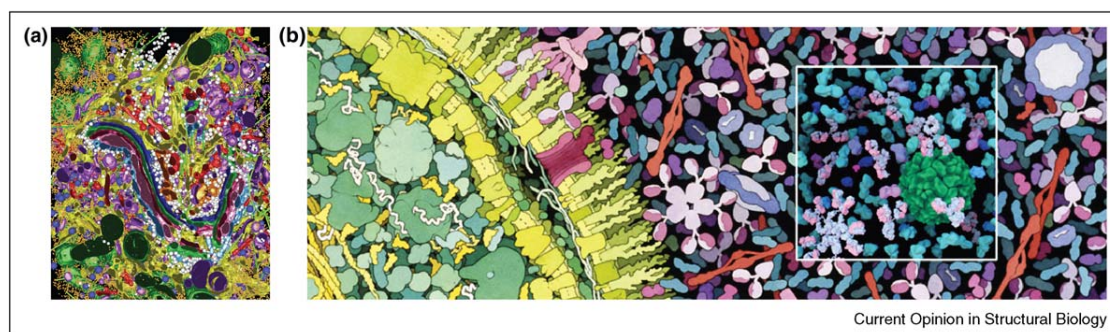
Experimental and artistic representations of the cellular interior. **(a)** 3D model of a cryo-electron tomography image of the Golgi region of an insuline-secreting HIT-T15 cell. The Golgi complex with its cisternae is shown in the center of the image (color coded in light blue, pink, cherry red, green and dark blue). The Golgi is displayed in the context of all surrounding organelles, vesicles, ribosomes, and microtubules: endoplasmic reticulum (ER), yellow; membrane-bound ribosomes, blue; free ribosomes, orange; microtubules, bright green; dense core vesicles, bright blue; clathrin-negative vesicles, white; clathrin-positive compartments and vesicles, bright red; clathrin-negative compartments and vesicles, purple; mitochondria, dark green. Reproduced courtesy of Dr Brad Marsh, The University of Queensland, Australia (adapted from [56]). **(b)** Artistic representation of an *E. coli* cell (cellular interior in light green, cell membrane in yellow) in blood serum (pink to violet). The inset is a three-dimensional model created from experimentally determined protein structures. Serum albumin is shown in turquoise. Y-shaped molecules and the large complex at lower left are antibodies. A poliovirus particle is depicted in green. Image courtesy of Dr David S. Goodsell and Arthur J. Olson, Scripps Institute, La Jolla, USA.

create, to varying degrees, artificial situations that are in-cell, but not strictly speaking *in vivo*.

In-cell NMR in prokaryotic cells

All prokaryotic in-cell NMR applications today employ the same rationale of sample over-expression, isotope labeling and in-cell NMR recordings within the same cell type, as was initially described in the first high-resolution in-cell NMR study in bacterial cells [11]. The basic principle of prokaryotic in-cell NMR methods relies on the fact that recombinant protein production via strong promoters like that of the bacteriophage T7 leads to the rapid intracellular accumulation of large amounts of plasmid-encoded proteins (Figure 2a). Switching the *Escherichia coli* cultures to isotope-labeled growth conditions, before recombinant protein induction, results in the selective incorporation of isotopes in the protein of interest only. Direct NMR recordings of such cultures thus yield high-resolution NMR signals of overexpressed, intracellular proteins. A number of reviews provide a comprehensive overview of prokaryotic in-cell NMR applications [7,12,13]. Specifically, recent bacterial in-cell NMR studies have probed protein–DNA interactions [14], intracellular protein dynamics [15], protein stability [16] and the structural *in vivo* features of many folded and intrinsically unfolded proteins [17,18,19••]. Solid-state NMR measurements of protein conformations inside bacterial inclusion bodies [20], the development of a flow-cell NMR bioreactor for in-cell NMR measurements [21], ^{19}F NMR studies on intracellular proteins [22], as well as *in vivo* incorporation schemes of non-natural amino acids for site-specific protein labeling and intracellular *in vivo* detection [23•], have also been reported.

Two ways in which in-cell methods have been applied in a more systematic manner are STINT-NMR (STructural INTeractions using NMR spectroscopy) and SMILI-NMR (Small Molecule Inhibitor Library by In-cell NMR). In STINT-NMR, the intracellular binding event of two sequentially expressed polypeptides is observed *in vivo* (Figure 2b). One of the two proteins is first produced in an isotope labeled form (i.e. under isotope-enriched growth conditions) and becomes the NMR-visible component. The other protein is expressed in an unlabeled form, after switching the medium to non-labeled growth conditions and thus remains NMR-invisible. Once the two proteins encounter each other in the cytoplasm of the expression host, the resulting binding event is analyzed in a manner similar to a high-resolution *in vitro* NMR titration experiment. Hence, *in vivo* resonance signals of amino acids that define the binding interface of the NMR-visible component display characteristic chemical shift changes upon increasing amounts of the additionally expressed, unlabeled ligand [24••,25]. This effect can be employed to comparatively analyze *in vitro* and *in vivo* binding behaviors of known interaction partners, or to test how small molecules interfere with cellular binding events. STINT-NMR was recently extended to probe the effects of post-translational modifications (PTMs) on the intracellular interaction characteristics of two sequentially expressed proteins. In order to do so, an inducible kinase domain that covalently modified the unlabeled STINT ligands was co-expressed with the protein of interest, and the modulated binding behavior analyzed by in-cell NMR spectroscopy [26•]. This concept was further extended by SMILI-NMR, where small molecules are screened for their ability to selectively modulate the intracellular binding behavior of two known

Figure 2
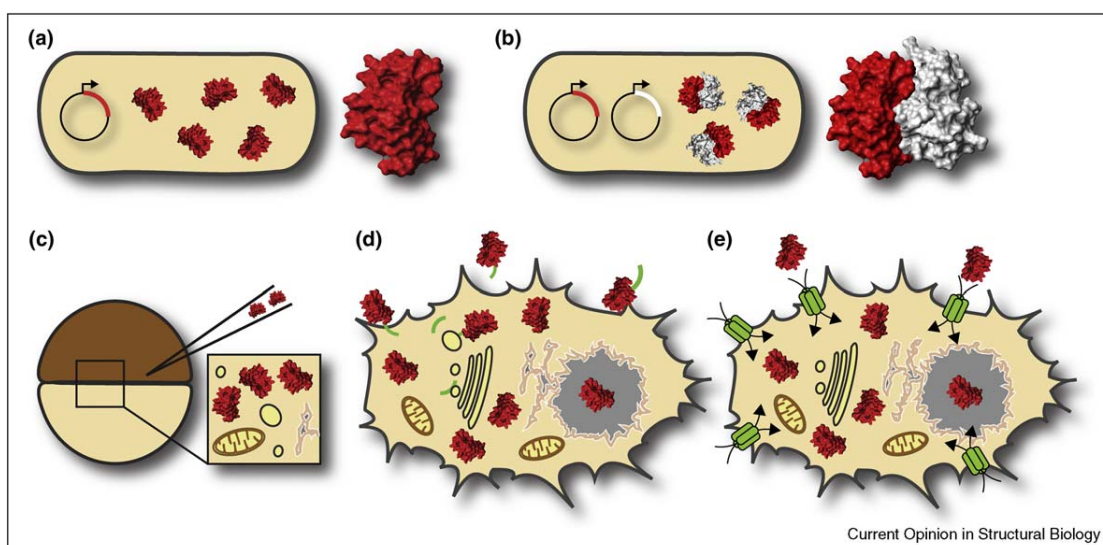
Schematic overview of the currently employed protocols for in-cell NMR sample preparations. **(a)** Prokaryotic in-cell NMR specimens are produced by recombinant protein production from strong plasmid promoters under isotope-labeled growth conditions. Accumulation of labeled proteins in the cytoplasm of the expression host enables direct NMR measurements on intact cells. **(b)** STINT-NMR methods rely on the sequential expression of two recombinant proteins under isotope-labeled (red) and non-labeled (white) growth conditions. Intracellular binding of the labeled component to the unlabeled ligand allows for NMR mapping of the *in vivo* binding interface. **(c)** Generation of in-cell NMR specimens in *Xenopus laevis* oocyte cells requires recombinant protein production and purification in bacterial cells and intracellular sample delivery by direct cytoplasmic microinjection. **(d)** In-cell NMR applications in mammalian cultured cells necessitate intracellular protein transduction procedures either via tagging of labeled cargo proteins (red) with cell-penetrating peptides (green) or **(e)** via cell-permeabilizing toxins (green) that enable the influx of isotope-labeled, non-modified, exogenous proteins (red).

interaction partners [27]. In that sense, STINT-NMR methods have collectively paved the way for high-resolution *in vivo* studies of intracellular protein–protein interactions.

Probably the biggest breakthrough in prokaryotic in-cell NMR spectroscopy was the de novo determination of a complete protein structure inside living *E. coli* cells [19••,28]. Even though various biomolecules had been studied in bacteria before, the intrinsic low sensitivity, high degree of line broadening and short *in vivo* half-lives of intracellular specimens had so far prevented the acquisition of extended NMR data sets for complete 3D structure determination. New developments in NMR methodology, reducing the overall time requirements for NMR experiments, in conjunction with combining NMR datasets of multiple in-cell NMR samples, enabled the solution of the *in vivo* structure of the metal-binding protein TTHA1718 from *Thermus thermophilus* HB8 (Figure 3). With the use of non-uniform, or nonlinear, sparse sampling schemes [29–31] and maximum-entropy reconstruction [32,33], data acquisition times were reduced from days to hours for the triple-resonance NMR experiments that are required for resonance assignments, as well as for 3D NOESY-type experiments for the collection of inter-proton distance restraints (Figure 3a and b). This eventually led to the calculation of an ensemble of in-cell NMR structures that converged to an excellent backbone root mean square deviation (rmsd) of 0.96 Å (Figure 3c). The *in vivo* structures displayed an overall backbone rmsd of 1.16 Å when compared to the *in vitro* determined structure of TTHA1718, thus proving that high-resolution protein structures can indeed be solved inside bacterial cells. Regions of TTHA1718 that differed from the *in vitro* structure included dynamic loop regions. These differences most likely occurred due to intracellular viscosity and macromolecular crowding. In addition, residues of the hypothetical metal-binding loop were severely line-broadened and hence were not detected by the in-cell NMR experiments. Binding of TTHA1718 to cellular metals is likely responsible for this behavior since mutant forms of the protein, in which all metal-coordinating residues had been replaced, showed no *in vivo* line broadening. With these successes, it is reasonable to suggest that the prokaryotic cytoplasm is an amenable environment for atomic-resolution structure determination.

In-cell NMR in eukaryotic cells

For in-cell NMR applications in eukaryotic cells, more convoluted sample preparation schemes are typically employed (Figure 2c, d, e). This results from the lack of appropriate eukaryotic cell systems for recombinant protein production and isotope labeling. Such systems

Figure 3
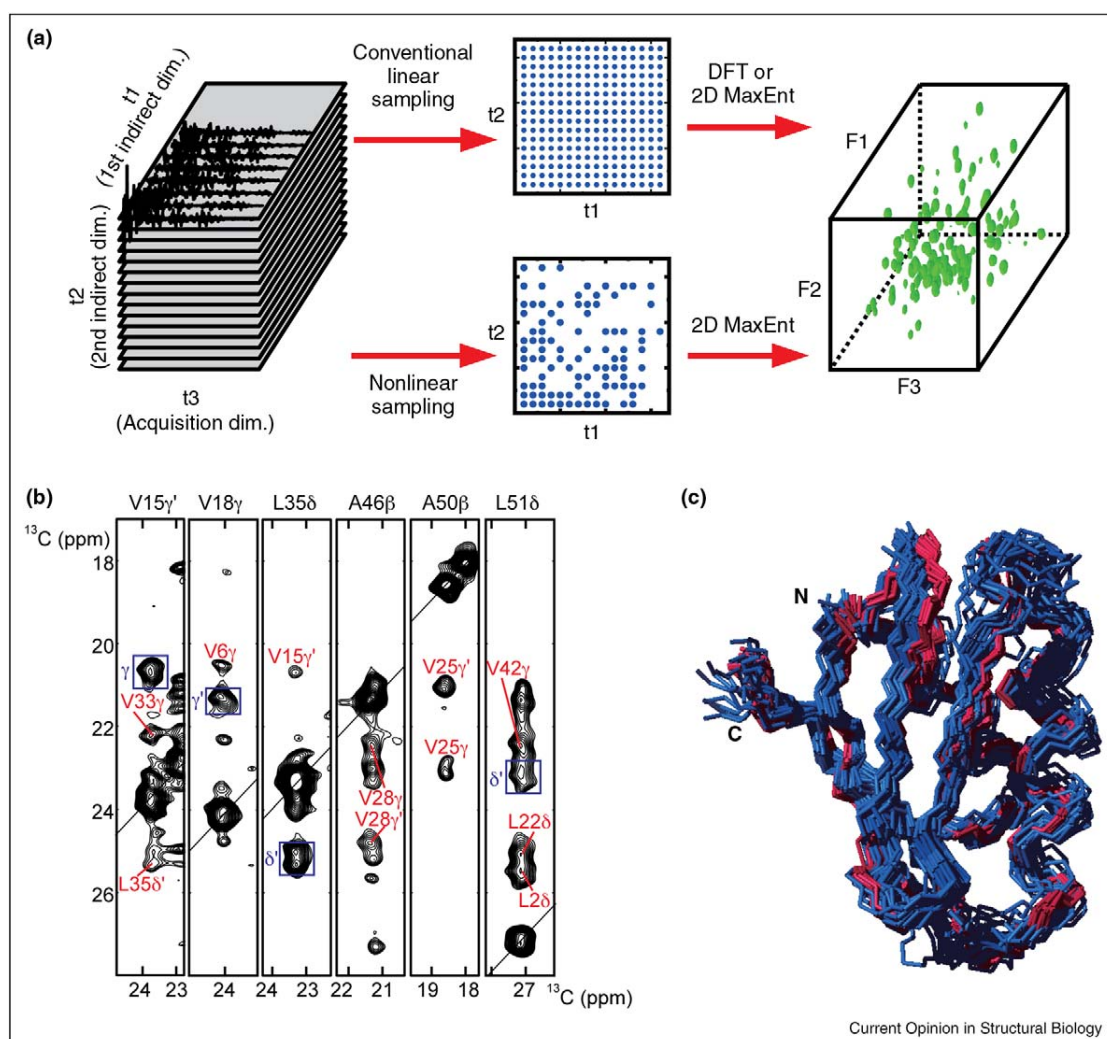
In vivo protein structure determination by in-cell NMR spectroscopy in prokaryotic cells. Rapid acquisitions of complete 3D data sets can be achieved by the implementation of nonlinear sampling schemes, discrete Fourier transformations (DFT) and maximum-entropy reconstructions (MaxEnt). A representation of those sampling and processing schemes is depicted in **(a)**. ^{13}C–^{13}C cross-sections of a 3D in-cell ^{13}C-separated HMQC-NOE-HMQC experiment **(b)**. A superposition of ensembles of *in vitro* (red) and *in vivo* (blue) determined structures of TTHA1718 illustrates their excellent overall convergence **(c)** (adapted from [16]).

would otherwise allow the preparation of in-cell NMR samples along the bacterial rationale (see above). In-cell NMR applications in *Xenopus laevis* oocytes for example, make use of isotope-labeled proteins that have been recombinantly produced in *E. coli* and that are then directly microinjected into oocyte cells (Figure 2c). Several successful studies employing this particular cell type and delivery strategy have been reported, including structural investigations of proteins [34**,35,36*] and nucleic acids [37]. With regard to eukaryotic in-cell NMR studies of an intrinsically disordered protein (IDP), one paper is of special interest [34**]. The group of Guy Lippens investigated the cellular *in vivo* properties of the human neuronal protein Tau, one of the largest known IDPs.

Tau exhibits disordered features over its entire protein length (441 amino acid residues, ~45 kDa) [38] and in contrast to other IDPs, has only weak propensities for transient secondary structures (turn conformations and β-strands) [39,40]. The biological function of Tau is to bind and stabilize microtubules. At the same time, it also constitutes a major component of intracellular, neuro-fibrillar tangles in Alzheimer's disease (AD) and displays a high tendency for aggregation [41]. The Tau protein thus represents, by any measure, a veritable challenge for NMR spectroscopy. It is very large by solution state NMR standards, displays a fairly low signal-to-noise despite being completely unfolded, has a confounding degree of spectral overlap and binds to one of the largest cellular

structures, microtubules, which are typically present at high natural abundance (∼10 μM). Most remarkably, Bodart *et al.* undertook to study Tau at close to physiological protein concentrations. At 5 μM intracellular Tau, in-cell NMR spectra revealed the preservation of its predominantly unfolded conformation. Due to the large degree of viscosity-driven NMR signal broadening, and the resulting increase in additional signal overlap, it was difficult to assess whether some portions of Tau were adopting novel structural features that were not present in the pure protein form. Overall, intracellular crowding did not appear to induce drastic conformational rearrangements, like it has been observed for other IDPs *in vivo* [42]. Several features of the in-cell NMR spectra did however indicate that large portions of Tau could be bound to cellular microtubules. This conclusion was drawn upon some striking similarities between *in vitro* NMR spectra of tubulin-bound Tau [43] and of *in vivo* established resonance signals of intracellular Tau. In addition, in-cell NMR spectra of Tau revealed NMR signals expected following phosphorylation by endogenous *Xenopus* kinases. Comparing these newly formed NMR resonances with those of *in vitro* phosphorylated Tau [38] confirmed that *in vivo* protein phosphorylation took place. This observation highlights one important feature of eukaryotic in-cell NMR measurements that we

have not yet touched upon. The unique ability to directly observe the establishment of post-translational protein modifications inside live cells. We have recently investigated this property in a more systematic manner for phosphorylation reactions in *Xenopus laevis* oocytes [44••] (Figure 4a). In our work, we analyzed the dynamic modification status of three independent phosphorylation sites: Two Casein kinase 2 (CK2), and one Cyclin-dependent protein kinase 1 (Cdk1) sites within the regulatory protein region of the viral SV40 large T antigen. Indeed, the time-resolved modification of all three sites, executed by endogenous protein kinases *in vivo*, could easily be 'followed' on a residue-specific basis and in a quantitative type of manner (Figure 4b). Even the stepwise phosphorylation of two neighboring serine residues by cellular CK2 could unambiguously be resolved in *Xenopus laevis* oocytes and cytostatic factor arrested (CSF) egg extracts. This could point to a new area for eukaryotic in-cell NMR research. *In situ* investigations of cellular PTM reactions at atomic resolution [45].

One of the biggest breakthroughs for eukaryotic in-cell NMR spectroscopy has been the ability to perform in-cell NMR measurements in mammalian cells. Recently, two alternative methods for intracellular protein delivery into immortalized human and monkey cells have been

Figure 4
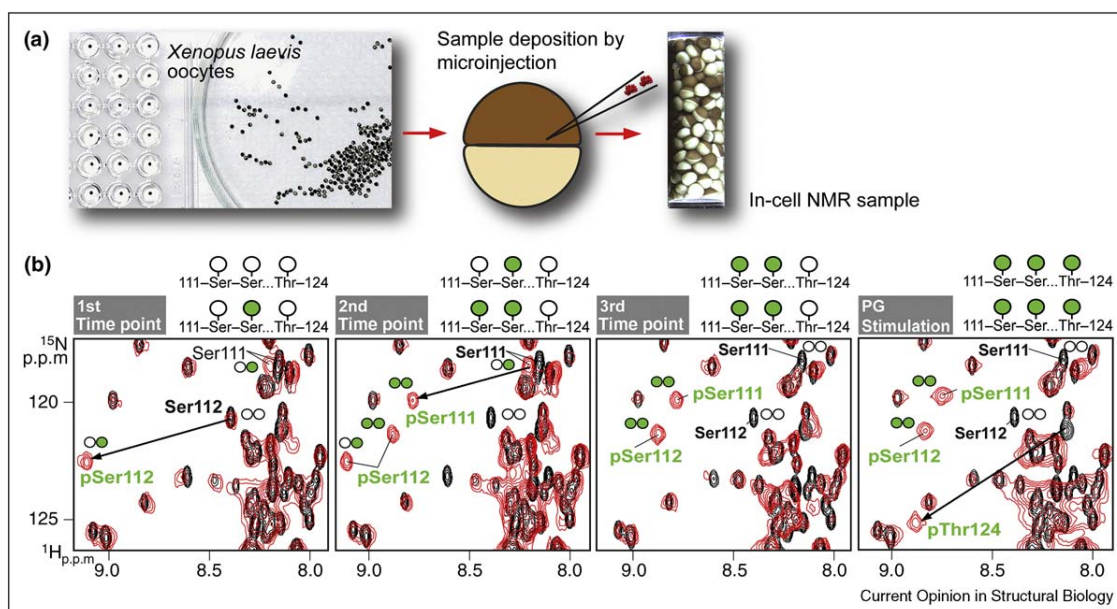
In-cell NMR measurements of cellular phosphorylation reactions in *Xenopus laevis* oocytes. **(a)** Isotope-labeled protein substrates are microinjected into interphase-arrested, stage VI oocytes. **(b)** Time-resolved NMR analysis reveals the stepwise modification of residues within the regulatory region of the viral SV40 large T antigen. Two neighboring Serines (Ser112 and Ser111) are phosphorylated by endogenous Casein kinase 2 (CK2). At initial time points, a mixed population of non-phosphorylated and mono-phosphorylated substrate molecules is detected. Cellular phosphorylation ensues until all protein species are modified at the more C-terminal PTM site. At intermediate times, mixed populations of mono-, and di-phosphorylated substrate molecules are present. At the final stage, all substrate molecules are modified at both sites. Upon progesterone (PG) stimulation, oocytes mature into fertilizable eggs. Concomitant, endogenous Cyclin-dependent kinase 1 (Cdk1) is activated. This leads to an additional phosphorylation event at Thre124 (adapted from [44]).

reported [46••,47•]. In the first instance, cell-penetrating peptide (CPP) tags were fused onto a recombinant, isotope labeled variant of human ubiquitin (Ub-3A), the *Streptococcal* protein G B1 domain (GB1) and FKBP12, in order to efficiently transduce those proteins into cultured HeLa and COS-7 cells [46••] (Figure 2d). Intracellular sample delivery via CPP tagging constitutes an appealing alternative to microinjection of mammalian cells (which, because of their small size, would need to be injected by the million) enabling the efficient manipulation of a large number of cells in a simple and straightforward manner. Upon intracellular 'cargo' delivery and CPP removal by action of an endogenous ubiquitin-specific C-terminal protease (DUB), or by intracellular reduction of a cysteine disulphide linker in between the CPP-moiety and the protein of interest, the authors managed to record several high-quality in-cell NMR spectra (Figure 5a). In one of their in-cell NMR experiments, Inomata *et al.* probed the binding behavior of externally administered FK506 and rapamycin to labeled, intracellular FKBP12 (Figure 5b). Two-dimensional $^1H/^{15}N$ correlation experiments unambiguously showed that identical sets of FKBP12 residues bound to FK506 and rapamycin *in vivo* and *in vitro*, indicating that both compounds crossed the cellular membrane and specifically bound to the intracellular target protein. This finding is especially significant with regard to pharmacological applications to monitor cellular drug–protein interactions. It exemplifies the usefulness of in-cell NMR measurements in determining pharmacokinetic properties like membrane permeating efficiency,

cytotoxicity, intracellular compound stability and availability, and for establishing cellular IC_{50} values in a straightforward manner.

In the second example, isotope-labeled thymosin β4 (Tβ4) protein was delivered into human 293F cells by means of the pore-forming toxin streptolysin O (SLO) [47•] (Figure 2e). Upon addition of Ca^{2+}, to re-seal the plasma membrane, intracellular Tβ4 was detected by high-resolution in-cell NMR experiments. One attractive feature of this approach, in contrast to the CPP strategy outlined above, is that no modifications to the protein are required for intracellular sample delivery. In-cell NMR spectra are thus uncompromised by CPP tags or by additional amino acids that remain present after intracellular tag removal. In their paper, Ogino *et al.* demonstrated that the intracellular protein underwent N-terminal acetylation, a cytoplasmic modification known to affect Tβ4. Intracellular protein delivery and the true in-cell status of the sample were thus confirmed by action of an intracellular enzyme. One disadvantage of the above delivery scheme is the requirement for mammalian cells that grow in suspension and for highly soluble target proteins. Because the amount of delivered sample depends on passive diffusion through the toxin pore, and not on an active uptake process like in the CPP case, the protein concentration gradient across the cell membrane directly relates to the overall transduction efficiency. Hence, high amounts of isotope-labeled proteins are needed (1 mM in the present study) in order

Figure 5
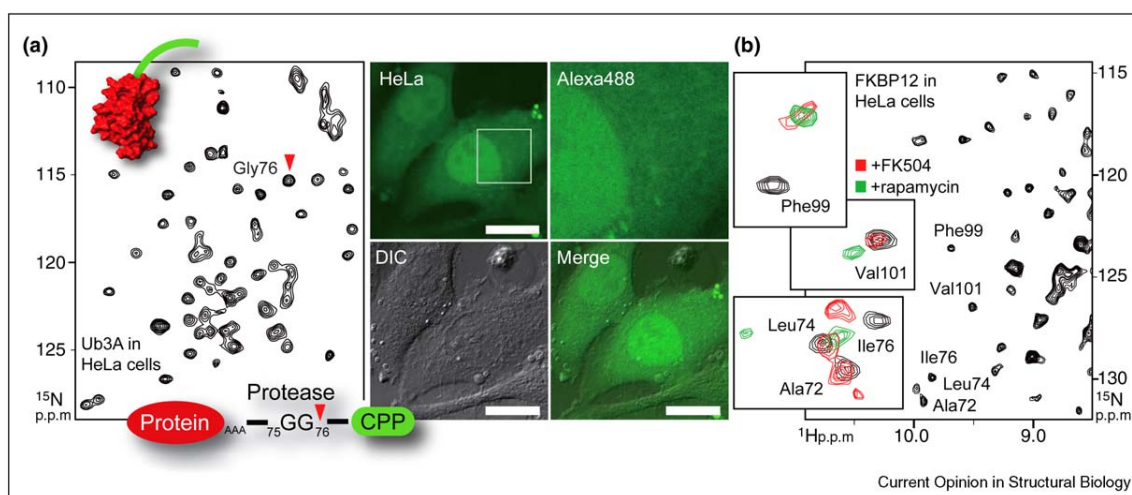
In-cell NMR measurements in human HeLa cells. A selected region of the $^1H/^{15}N$ 2D correlation spectrum of intracellular Ub3A is shown in **(a)**. The labeled protein (red) is released from the CPP moiety (green) by action of an endogenous protease. The newly formed C-terminus of Ub3A gives rise to the appearance of the Gly76 NMR signal at the characteristic resonance frequency (indicated by a red arrowhead). Intracellular CPP cleavage yields a uniform cytoplasmic distribution of Ub3A, as indicated by fluorescence microscopy data of the Alexa-labeled protein (middle panel). A selected view of intracellular FKBP12 is shown in **(b)**. *In vivo* binding of FK504 (red), or rapamycin (green), to FKBP12 is indistinguishable from the equivalent *in vitro* reactions. In-cell chemical shift changes of FKBP residues Phe99, Val101, Ile76, Leu74 and Ala72, upon compound binding are shown in the respective close-up regions (adapted from [46••]).

to yield intracellular protein concentrations in the low micromolar range. If adherent cells were to be targeted, substantially higher volumes of 'delivery medium' and thus higher overall concentrations of labeled proteins would be required, simply because cells could no longer be manipulated in small volumes.

To date, mammalian in-cell NMR applications have not yet yielded their full biological impact. They have nevertheless proven one essential point: High-resolution in-cell NMR studies in cultured mammalian cells are indeed possible. It is with great excitement that we await more biologically oriented investigations that are likely to follow suit.

Concluding remarks

Despite the fact that in-cell NMR methods offer many intriguing possibilities for cellular *in vivo* studies, the technique bears several shortcomings. As is often stated, the biggest problems of all current in-cell NMR applications are the high intracellular protein concentrations at which these experiments are being performed (typically μM to mM). This range of protein concentrations is clearly above the physiologically relevant limit. As such, and despite the fact that in-cell NMR experiments are conducted on proteins inside live cells, in-cell NMR measurements hardly justify the notion of real *in vivo* investigations. So why do we not perform in-cell NMR recordings at lower, more physiologically relevant protein levels? The answer to this question lies in the intrinsic low sensitivity of high-resolution NMR methods. At high intracellular protein concentrations, the average signal-to-noise of most NMR experiments is of sufficient quality to perform them fairly quickly (i.e. with a small number of scans/transients). Because this reduces the adverse effects of prolonged cellular exposure to the unfavorable conditions of in-cell NMR measurements (typically lack of replenishment with nutrients, diminished aeration, etc.), high intracellular protein levels are often desired. At the same time, severe sample over-expression, or the delivery of unnaturally large amounts of 'foreign' proteins can lead to unspecific binding, non-physiological aggregation events, or induced cellular toxicity. Despite the fact that all of these scenarios have been experimentally observed [15,48], high intracellular protein concentrations have long been considered unavoidable in in-cell NMR measurements. Here, one type of methodological NMR advancement has truly provided a quantum leap for in-cell NMR applications: The development of ultrafast NMR methods for two-dimensional, and three-dimensional NMR experiments [49–51]. With those tools at hand, the time requirements for NMR experiments have been reduced from tens-of-minutes to seconds, for 2D NMR experiments, and from days to hours in the case of 3D NMR experiments. In combination with the aforementioned sparse sampling techniques, full 3D datasets can even be recorded within minutes. Without them,

several of the most demanding in-cell NMR applications would not have been possible [21,34,37,38,46••]. The availability of rapid NMR methods can be exploited in two ways. Either to reduce the required in-cell NMR sample concentrations, performing more NMR scans within the same amount of experimental time for comparable signal intensities, or to employ high protein concentrations and to achieve the same S/N in a fraction of the previously required experimental time. In practice, most future in-cell NMR applications will settle on a compromise between the two options, but in principle, in-cell NMR measurements at low, physiological protein concentrations have become experimentally feasible.

Another in-cell NMR problem is that of sample leakage. Two papers on in-cell protein dynamics had to be retracted when it became clear that the measured NMR signals did not in fact originate from intracellular proteins [52–54]. The problem of protein leakage has to be dealt with in an upfront manner and several measures can be employed in order to avoid it [12]. Sedimentation of bacterial and mammalian cells after measurements and detection of potential leakage signals in their supernatants have become routine procedures for ruling out extracellular sample 'spills' during in-cell NMR experiments. Viability tests after in-cell NMR measurements, to evaluate the true *in vivo* status of the in-cell NMR specimens, are also routinely employed. For NMR applications in *Xenopus* oocytes, co-injection of fluorescent dyes [55] and the usage of a minimal incision robotic injection device [36•] have been reported. Protein transduction protocols for sample delivery into adherent mammalian cells fortuitously include a protease treatment step, required in order to detach mammalian cells from their culture flasks. A side effect of this 'digestive' procedure is to ensure that NMR signals from improperly internalized, extracellular protein molecules are ablated, so that they cannot bias in-cell NMR experiments. These measures are bound to improve the quality of in-cell NMR data, as well as increase the confidence in in-cell NMR results. More importantly, it remains our responsibility to win back the trust of the biological community by the rigorous implementation of more stringent control experiments, and by a more considerate attitude towards the communication of in-cell NMR findings. Despite these reservations, the stage is now set for in-cell NMR spectroscopy to leave behind its awkward teenage years and to mature into a biophysical tool of general applicability.

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