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In-cell NMR: from target structure and dynamics to drug screening

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Abstract

The cellular environment can affect the structure and function of pharmacological targets, and the interaction with potential drugs. Such complexity is often overlooked in the first steps of drug design, where compounds are screened and optimized in vitro, leading to high failure rates in the pre-clinical and clinical tests. In-cell NMR spectroscopy has the potential to fill this gap, as it allows structural studies of proteins and nucleic acids directly in living cells, from bacteria to human-derived, providing a unique way to investigate the structure and dynamics of ligand-target interactions in the native cellular context. When applied to drug screening, in-cell NMR provides insights on binding kinetics and affinity towards a cellular target, offering a powerful tool for improving drug potency at an early stage of drug development.

Introduction

Structural knowledge of biological macromolecules is fundamental for understanding their function and for developing more effective drugs. However, structural studies on pharmacological targets are typically carried out in vitro, where any possible influence of the physiological environment is lost. Indeed, much more reliable information would be obtained if cellular targets could be characterized in the complex environment of the cell membrane or the intracellular milieu. Ultimately, this unaccounted complexity is among the causes of the high attrition rate in modern drug design campaigns, where promising ligands selected in vitro often show poor activity and/or fail to engage the right target within the cells. To optimize drug candidates to be effective in the complex native environment of the target, ligand-target interaction studies should be performed directly in living cells. Among the structural biology techniques, NMR spectroscopy is the most suited to obtain atomic-level structural insights on the interaction between a ligand and its target, and to probe the dynamics

and kinetics of such process, in a non-destructive way [1]. More importantly, NMR can analyze ligands and macromolecules in complex and heterogeneous environments. Cell lysates/extracts have been used for NMR studies in close-to-native conditions [2,3]. However, lysates cannot retain the complexity of intact cells, as the molecular constituents lose their spatial organization, are mixed together and diluted, resulting in the loss of emergent properties such as compartmentalization, crowding, quinary interactions, and homeostasis of metabolites and ions [4]. To retain these properties, the NMR analysis should be carried out in the interior (or at the surface) of intact, living cells. Over the last two decades, continuous development of cellular NMR approaches has greatly expanded the capability of NMR to probe macromolecular structure [5,6], dynamics [7,8], maturation and interactions with cofactors, cellular partners and external molecules [9] directly in intact cells. The history of cellular NMR in its many flavors and applications has been extensively covered in other recent reviews [10–14]. Here, we focus on the recent developments of in-cell NMR (Figure 1A) applied to the characterization of drug-target complexes in intact cells/membranes and to the screening of drug candidates towards a specific cellular target (Figure 1B). We show that, thanks to the latest methodology and hardware advancements, in-cell NMR spectroscopy can now probe within living cells target engagement, conformational changes upon complex formation, and binding kinetics and thermodynamics, offering a potentially revolutionizing tool in the development of more effective drugs against cellular targets.

Structural and dynamical changes upon binding

Interactions of pharmacological targets with ligands or partners induce changes in conformation and dynamics, which causes changes in the frequency and relaxation of the NMR signals of the affected nuclei. Indeed, backbone Chemical Shift Perturbation (CSP) is among the most commonly methods to probe protein-ligand and protein-protein interactions in solution [15]. When the backbone resonance assignment is available, CSP analysis offers a straightforward way to find out where a ligand binds sites on the surface of the protein, and the to assess the extent of ensuing structural rearrangements. *In vitro*, titration experiments allow routine determination of the interaction surface and the binding affinity of ligands or fragments [15]. Since the first proof that isotopic labeling allowed selective investigation of a protein of interest in intact cells by heteronuclear NMR [16], it was immediately realized that protein-ligand interactions could be easily monitored in *E. coli* through the CSP induced by ligand binding [17]. Backbone CSP were later employed to probe protein-ligand interactions in various types of cells [18–21**]. To date, CSP analysis remains one of the most information-rich experiments when studying macromolecular chemical and conformational changes and interactions

by in-cell NMR, and is at the basis of the latest drug screening applications (see below). While easy to measure, chemical shifts differences can only be determined when both the free and bound states of a protein give rise to visible signals in the NMR spectrum. However, it often happens that soluble intracellular targets interact with other cellular macromolecules, causing a drastic decrease in tumbling rate and, as a consequence, broadening their NMR signals beyond detection [22–25]. Importantly, these interactions may be modulated by ligand binding, if the ligand interferes with the interaction surface between macromolecules. Under such circumstances, it is still possible to monitor the effect of a ligand on an intracellular target, based on the change of signal intensity upon ligand treatment. Shekhtman and coauthors relied on this approach to monitor the ‘indirect’ effect of ligands that, upon binding to their intracellular target (antibiotics binding to the ribosome), either liberate the observed molecule (thioredoxin) or trigger its interaction with a third component (RNA), with opposite effects on the intensity of the observed signals [26,27]. While this approach is ingenious, care must be taken when interpreting the results: the assay gives meaningful results only if the signal intensity changes are caused by the effect of the ligand on the target molecule or its partners, and not by other unrelated mechanisms. Similar to proteins, chemical shift changes upon complex formation are determinant to study the interaction between ligands and small DNA/RNA targets in cells. The Trantirek group pioneered this application, showing that the ^1H spectra of small DNA motifs (a 24-nt hairpin and a 11-bp DNA duplex) delivered to the nucleus of human cells (Figure 2A) change dramatically when the same molecules are complexed with ligands, making possible to determine whether the complexes are stable in the nuclear environment (Figure 2B) [28]. Trantirek, Schwalbe and coauthors further extended the approach to observe the structural rearrangement of functional short riboswitch aptamers upon binding of their cognate ligand, 2'-deoxyguanosine, in human cells [29**]. Compared to DNA, RNA molecules are even more challenging due to fast degradation in the cellular environment. With the aid of isotope labeling and 2D heteronuclear NMR, large functional RNA riboswitches (up to 70-nt) can be observed in *Xenopus laevis* oocytes, without resorting to chemical modification, whereas lower delivery efficiencies in human cells restrict the approach to smaller RNA molecules (~15-nt).

Ligand-target complexes in frozen cells/membranes

As mentioned above, the slow tumbling of macromolecules due to interactions with the environment or to exceedingly large molecular sizes make several targets unamenable to solution NMR analysis. The same holds true for proteins embedded in cellular membranes, which completely restrict their rotational motion. To overcome this limitation, cellular/native membrane solid-state NMR (SSNMR)

approaches have been developed [13]. High-resolution SSNMR relies on fast spinning of the sample (at the so-called magic angle with respect to the static magnetic field) to recover narrow spectral features resembling those of solution NMR. Furthermore, Dynamic Nuclear Polarization (DNP)-assisted SSNMR can greatly enhance the signals of interest in cryogenically cooled samples of intact cells or membranes. The approach, first introduced by the Baldus group [30,31], was recently applied by Weingarth and coauthors to structurally characterize the mode of binding of two antimicrobial peptides, nisin and teixobactin, to their native target lipid-II within membranes from *Micrococcus flavus* [32,33**]. In the native membrane environment, both compounds form pore-like complexes with lipid-II that markedly differ from those obtained in synthetic micelles, revealing structural details that are crucial for understanding the mechanism of action of this promising class of antibiotics and for designing novel, more effective antimicrobial compounds. Baldus and coauthors showed that DNP-assisted SSNMR on cryogenically cooled cells allows the observation of cytoplasmic proteins involved in interactions that would prevent solution NMR studies [34]. Petzold and coauthors employed the same approach to observe the signals of an antisense oligonucleotide drug in frozen human cells, likely involved in macromolecular complexes with its target mRNA and/or with other cellular components [35]. While the DNP-SSNMR application to intact cells is still in its infancy, the above works represent the first steps towards its broader application to characterize intracellular ligand-target complexes.

Drug screening by in-cell NMR

As seen above, in-cell NMR can directly probe the interaction between ligands and their intracellular targets. This capability makes it an extremely appealing tool in the field of drug development, which suffers from high attrition rates. Indeed, most of the compounds optimized for maximum in vitro activity fail to show activity in cellular or animal models, or worse, do not pass the clinical trials due to poor efficacy or selectivity towards the target. To improve the success rate of the last steps, compounds highly active in vitro that cannot engage their intracellular target should be identified as early as possible. Cellular assays often rely on downstream effects, such as cell proliferation or invasiveness and, because do not provide insights on the mode of action, they are prone to false positives. In extreme cases, compounds could be selected that exert the desired effect through a completely unrelated mechanism, causing it to fail in the following phases due to poor activity or toxicity in vivo. Biochemical methods have been proposed to probe target engagement in cells, such as the cellular thermal shift assay and its variants [36]. While powerful and high-throughput, these assays are extremely ligand- and target-dependent, as they rely on different temperature-dependent unfolding of the free and bound target, and must be interpreted with caution [37].

Following the first proof-of-concept works on protein-ligand interactions [17,18], in-cell NMR has been successfully applied to drug screening. Shekhtman and coauthors devised an approach, SMILI NMR, to screen libraries of compounds for protein binding in bacteria by relying on either CSP or changes in the intensity of the target protein signals (Figure 3A, 3B) [19]. To increase the throughput, the authors proposed a matrix approach, where a compound library arranged in a $N \times M$ matrix is screened on $N + M$ cell samples, each treated with a mixture of N or M compounds. This strategy led to the identification of three compounds that efficiently inhibited a protein-protein interaction (a notoriously challenging target in drug development), namely that of the prokaryotic ubiquitin like protein (Pup) with the mycobacterial proteasome ATPase (Mpa), showing potential implications in the development of novel antimicrobial drugs (Figure 3C, 3D) [38]. These works suggested that in-cell NMR could combine the best of two worlds: the direct proof of binding typical of in vitro screenings, and the high biological relevance of the cellular environment (Figure 3E). With the same rationale, our research group applied in-cell NMR to the screening of drugs in human cells overexpressing the target protein of interest [21**]. By protein-observed in-cell NMR, we analyzed the dose- and time-dependent binding of a set of compounds to the first two isoforms of the human carbonic anhydrase (CA I and II), part of a family of pharmacological targets for glaucoma, epilepsy, cardiovascular diseases, and cancer (Figure 4A). That work showed that, for nanomolar-affinity compounds, cell membrane permeability becomes the bottleneck that determines whether they will bind the intracellular target (Figure 4B), with striking correlation with the efficacy in vivo. Time-resolved in-cell NMR analysis over several hours (Figure 4C) confirmed that drugs with similar affinities bind intracellular CA II with very different rates, as a function of cell permeability [39*]. We further investigated the binding kinetics and stability of existing drugs to CA II in human cells, and classified them based on their binding kinetics as either 1) fast, stable binders; 2) slow, stable binders and 3) unstable binders, which slowly left CA II over time [40]. Intriguingly, the latter class comprised drugs developed for other targets, suggesting that the binding instability is caused by the presence of multiple high-affinity targets. The same approach allows measuring intracellular binding affinities in the nanomolar range, by means of in-cell competition binding experiments where cells are incubated with a test compound at variable concentration and a reference with known affinity at constant concentration (Figure 4D, 4E) [41]. At the diffusion-limited equilibrium, the affinity of the test compound is determined by the fraction of protein bound to each ligand (Figure 4F). The Trantirek group applied a similar approach to screen ligands towards an intracellular DNA G-quadruplex using ^{19}F in-cell NMR [42*]. ^{19}F is a highly sensitive nucleus that, once chemically introduced on the molecule of interest, provides an excellent background-free probe to investigate macromolecules in living cells [43,44] and to distinguish free and ligand-bound forms.

Ligand-observed cellular NMR

Specular to protein-observed approaches, ligand-observed NMR is widely used *in vitro* for high-throughput screening, thanks to faster analysis through ^1H NMR and lower sample preparation costs. When applied to cells, ligand observation faces additional challenges, due to 1) the difficulty of observing the signals of a small organic molecule bound to its target in the cellular environment, without resorting to isotope labeling (which requires *ex-novo* organic synthesis) and 2) the lack of information on *where* the compound is binding in the cellular context. Despite these limitations, several applications of ligand-observed NMR in intact cells have been reported. Due to the first issue, ligand-observed NMR is better suited to probe binding to proteins on the plasma membrane of intact cells (on-cell NMR) by relying on saturation transfer difference experiments, which detect intensity changes on the free external ligand upon interaction [45–47], and transferred nuclear Overhauser effect, which provides additional information on the ligand binding mode [48,49]. Both approaches require carefully designed control experiments to demonstrate binding to a specific target. Lastly, Primikyri et al. extended the approach to screen an intracellular target, Bcl-2, in human cells, thus widening the range of applications of ligand-observed in-cell NMR [50].

Outlook: present and future

The cellular NMR approaches described above provide precious structural and thermodynamic insights on ligand-target interactions in living cells, and allow screening of lead compounds for binding in cellular settings, holding great promises for the development of future drugs. Solid-state NMR has a great potential for structural characterization, as it is not limited by the tumbling rate of protein-ligand adducts, while solution NMR approaches can probe the kinetics and dynamics of ligand binding at physiological temperatures. The most recent applications of in-cell NMR to drug screening show that protein-observed in-cell drug screening, despite being low-throughput, offers unique advantages in terms of selectivity, thanks to the exquisite chemical sensitivity of NMR. Indeed, because each protein-ligand adduct exhibits different chemical shifts, nanomolar affinity constants can be measured by competition binding with accuracy and specificity unmatched, to our knowledge, by any other live-cell assay. On the other hand, ligand-observed approaches are preferable in terms of throughput and cost-per-sample and are less constrained in terms of types of cells and target expression levels, therefore they will likely see a broader application to drug screening in cells. The recent advancements in terms of hardware will further extend the capabilities of NMR: ultra-high field spectrometers

provide higher sensitivity and resolution [51], and improved NMR bioreactor designs now allow time-resolved NMR on living cells lasting several days [39*,52,53]. Finally, we envision that the next generation of cellular NMR approaches will rely upon novel spectroscopic tools, such as the multidimensional homo- and heteronuclear ^{19}F NMR experiments recently reported [54,55], which will allow higher-throughput and -content ligand screening and the structural investigation of more challenging cellular targets.

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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. Pellecchia M, Bertini I, Cowburn D, Dalvit C, Giralt E, Jahnke W, James TL, Homans SW, Kessler H, Luchinat C, et al.: **Perspectives on NMR in drug discovery: a technique comes of age.** *Nat Rev Drug Discov* 2008, **7**:738–745.
2. Theillet F-X, Smet-Nocca C, Liokatis S, Thongwichian R, Kosten J, Yoon M-K, Kriwacki RW, Landrieu I, Lippens G, Selenko P: **Cell signaling, post-translational protein modifications and NMR spectroscopy.** *J Biomol NMR* 2012, **54**:217–236.
3. Dalvit C, Veronesi M, Vulpetti A: **Fluorine NMR functional screening: from purified enzymes to human intact living cells.** *J Biomol NMR* 2020, **74**:613–631.
4. Zhou H-X, Rivas G, Minton AP: **Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences.** *Annual Review of Biophysics* 2008, **37**:375–397.
5. Sakakibara D, Sasaki A, Ikeya T, Hamatsu J, Hanashima T, Mishima M, Yoshimasu M, Hayashi N, Mikawa T, Wälchli M, et al.: **Protein structure determination in living cells by in-cell NMR spectroscopy.** *Nature* 2009, **458**:102–105.
6. Tanaka T, Ikeya T, Kamoshida H, Suemoto Y, Mishima M, Shirakawa M, Güntert P, Ito Y: **High-Resolution Protein 3D Structure Determination in Living Eukaryotic Cells.** *Angew Chem Int Ed Engl* 2019, **58**:7284–7288.
7. 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**:e10027.
8. Theillet F-X, Binolfi A, Bekei B, Martorana A, Rose HM, Stuver M, Verzini S, Lorenz D, van Rossum M, Goldfarb D, et al.: **Structural disorder of monomeric α -synuclein persists in mammalian cells.** *Nature* 2016, **530**:45–50.
9. Luchinat E, Banci L: **In-Cell NMR in Human Cells: Direct Protein Expression Allows Structural Studies of Protein Folding and Maturation.** *Acc Chem Res* 2018, **51**:1550–1557.
10. Siegal G, Selenko P: **Cells, drugs and NMR.** *J Magn Reson* 2019, **306**:202–212.
11. Kang C: **Applications of In-Cell NMR in Structural Biology and Drug Discovery.** *Int J Mol Sci* 2019, **20**:139.
12. Yamaoki Y, Nagata T, Sakamoto T, Katahira M: **Recent progress of in-cell NMR of nucleic acids in living human cells.** *Biophys Rev* 2020, **12**:411–417.
13. Narasimhan S, Folkers GE, Baldus M: **When Small becomes Too Big: Expanding the Use of In-Cell Solid-State NMR Spectroscopy.** *ChemPlusChem* 2020, **85**:760–768.

14. Luchinat E, Cremonini M, Banci L: **Radio Signals from Live Cells: The Coming of Age of In-Cell Solution NMR.** *Chem Rev* 2022, doi:10.1021/acs.chemrev.1c00790.
 15. Williamson MP: **Using chemical shift perturbation to characterise ligand binding.** *Progress in Nuclear Magnetic Resonance Spectroscopy* 2013, **73**:1–16.
 16. Serber Z, Dötsch V: **In-cell NMR spectroscopy.** *Biochemistry* 2001, **40**:14317–14323.
 17. Hubbard JA, MacLachlan LK, King GW, Jones JJ, Fosberry AP: **Nuclear magnetic resonance spectroscopy reveals the functional state of the signalling protein CheY in vivo in Escherichia coli.** *Molecular Microbiology* 2003, **49**:1191–1200.
 18. Inomata K, Ohno A, Tochio H, Isogai S, Tenno T, Nakase I, Takeuchi T, Futaki S, Ito Y, Hiroaki H, et al.: **High-resolution multi-dimensional NMR spectroscopy of proteins in human cells.** *Nature* 2009, **458**:106–109.
 19. Xie J, Thapa R, Reverdatto S, Burz DS, Shekhtman A: **Screening of small molecule interactor library by using in-cell NMR spectroscopy (SMILI-NMR).** *J Med Chem* 2009, **52**:3516–3522.
 20. 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.
 21. Luchinat E, Barbieri L, Cremonini M, Nocentini A, Supuran CT, Banci L: **Drug Screening in Human Cells by NMR Spectroscopy Allows the Early Assessment of Drug Potency.** *Angew Chem Int Ed Engl* 2020, **59**:6535–6539.
- ** First protein-observed drug screening by in-cell NMR in human cells. Chemical shift perturbation reveals the binding of drugs and lead compounds to the active site of a cytosolic protein, CA II. Dose- and time-dependent analysis reveals that the ligand diffusion across the membrane is the slow step, which strongly correlates with the potency of approved drugs.
22. Barnes CO, Monteith WB, Pielak GJ: **Internal and global protein motion assessed with a fusion construct and in-cell NMR spectroscopy.** *Chembiochem* 2011, **12**:390–391.
 23. Crowley PB, Chow E, Papkovskaia T: **Protein interactions in the Escherichia coli cytosol: an impediment to in-cell NMR spectroscopy.** *Chembiochem* 2011, **12**:1043–1048.
 24. Luh LM, Hänsel R, Löhr F, Kirchner DK, Krauskopf K, Pitzius S, Schäfer B, Tufar P, Corbeski I, Güntert P, et al.: **Molecular crowding drives active Pin1 into nonspecific complexes with endogenous proteins prior to substrate recognition.** *J Am Chem Soc* 2013, **135**:13796–13803.
 25. Barbieri L, Luchinat E, Banci L: **Protein interaction patterns in different cellular environments are revealed by in-cell NMR.** *Sci Rep* 2015, **5**:14456.
 26. DeMott CM, Majumder S, Burz DS, Reverdatto S, Shekhtman A: **Ribosome Mediated Quinary Interactions Modulate In-Cell Protein Activities.** *Biochemistry* 2017, **56**:4117–4126.
 27. Breindel L, DeMott C, Burz DS, Shekhtman A: **Real-Time In-Cell Nuclear Magnetic Resonance: Ribosome-Targeted Antibiotics Modulate Quinary Protein Interactions.** *Biochemistry* 2018, **57**:540–546.

28. Krafcikova M, Dzatko S, Caron C, Granzhan A, Fiala R, Loja T, Teulade-Fichou M-P, Fessler T, Hänsel-Hertsch R, Mergny J-L, et al.: **Monitoring DNA-Ligand Interactions in Living Human Cells Using NMR Spectroscopy.** *J Am Chem Soc* 2019, **141**:13281–13285.
29. Broft P, Dzatko S, Krafcikova M, Wacker A, Hänsel-Hertsch R, Dötsch V, Trantirek L, Schwalbe H: **In-Cell NMR Spectroscopy of Functional Riboswitch Aptamers in Eukaryotic Cells.** *Angew Chem Int Ed Engl* 2021, **60**:865–872.
- ** First in-cell NMR observation of the binding of cognate ligands to small RNA motifs (functional riboswitch aptamers) in *Xenopus laevis* oocytes and in human cells. The approach allows the structural characterization of much larger (70 nt) RNA fragments than previously reported, without the need for chemical modification to increase RNA stability.
30. Renault M, Pawsey S, Bos MP, Koers EJ, Nand D, Tommassen-van Boxtel R, Rosay M, Tommassen J, Maas WE, Baldus M: **Solid-state NMR spectroscopy on cellular preparations enhanced by dynamic nuclear polarization.** *Angew Chem Int Ed Engl* 2012, **51**:2998–3001.
31. Kaplan M, Narasimhan S, de Heus C, Mance D, van Doorn S, Houben K, Popov-Čeleketić 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.
32. Medeiros-Silva J, Jekhmane S, Paioni AL, Gawarecka K, Baldus M, Swiezewska E, Breukink E, Weingarh M: **High-resolution NMR studies of antibiotics in cellular membranes.** *Nat Commun* 2018, **9**:3963.
33. Shukla R, Medeiros-Silva J, Parmar A, Vermeulen BJA, Das S, Paioni AL, Jekhmane S, Lorent J, Bonvin AMJJ, Baldus M, et al.: **Mode of action of teixobactins in cellular membranes.** *Nat Commun* 2020, **11**:2848.
- ** The binding mode of teixobactin, a promising antimicrobial peptide, is investigated in native cellular membranes using solid-state NMR. Teixobactin is found to bind its main target, Lipid II, weakly in cellular membranes, suggesting an additional mechanism to explain its high antimicrobial activity.
34. Narasimhan S, Scherpe S, Lucini Paioni A, van der Zwan J, Folkers GE, Ovaa H, Baldus M: **DNP-Supported Solid-State NMR Spectroscopy of Proteins Inside Mammalian Cells.** *Angewandte Chemie International Edition* 2019, **58**:12969–12973.
35. Schlagnitweit J, Friebe Sandoz S, Jaworski A, Guzzetti I, Aussenac F, Carbajo RJ, Chiarparin E, Pell AJ, Petzold K: **Observing an Antisense Drug Complex in Intact Human Cells by in-Cell NMR Spectroscopy.** *ChemBioChem* 2019, **20**:2474–2478.
36. Martinez Molina D, Nordlund P: **The Cellular Thermal Shift Assay: A Novel Biophysical Assay for In Situ Drug Target Engagement and Mechanistic Biomarker Studies.** *Annu Rev Pharmacol Toxicol* 2016, **56**:141–161.
37. Seashore-Ludlow B, Axelsson H, Lundbäck T: **Perspective on CETSA Literature: Toward More Quantitative Data Interpretation.** *SLAS DISCOVERY: Advancing the Science of Drug Discovery* 2020, **25**:118–126.
38. DeMott CM, Girardin R, Cobbert J, Reverdatto S, Burz DS, McDonough K, Shekhtman A: **Potent Inhibitors of Mycobacterium tuberculosis Growth Identified by Using in-Cell NMR-based Screening.** *ACS Chem Biol* 2018, **13**:733–741.

39. Luchinat E, Barbieri L, Campbell TF, Banci L: **Real-Time Quantitative In-Cell NMR: Ligand Binding and Protein Oxidation Monitored in Human Cells Using Multivariate Curve Resolution.** *Anal Chem* 2020, **92**:9997–10006.

* An improved NMR bioreactor design is applied to monitor protein-ligand interactions and protein disulfide bond formation in human cells. The bioreactor preserves cell viability for up to 72 h. Time-resolved in-cell NMR data are analyzed by multivariate curve resolution to obtain the concentration profile of the species of interest.

40. Luchinat E, Barbieri L, Cremonini M, Nocentini A, Supuran CT, Banci L: **Intracellular Binding/Unbinding Kinetics of Approved Drugs to Carbonic Anhydrase II Observed by in-Cell NMR.** *ACS Chem Biol* 2020, **15**:2792–2800.

41. Luchinat E, Barbieri L, Cremonini M, Pennestri M, Nocentini A, Supuran CT, Banci L: **Determination of intracellular protein-ligand binding affinity by competition binding in-cell NMR.** *Acta Crystallogr D Struct Biol* 2021, **77**:1270–1281.

42. Krafčík D, Ištvaníková E, Džatko Š, Víšková P, Foldynová-Trantírková S, Trantírek L: **Towards Profiling of the G-Quadruplex Targeting Drugs in the Living Human Cells Using NMR Spectroscopy.** *International Journal of Molecular Sciences* 2021, **22**:6042.

* First application of in-cell ¹⁹F NMR to drug screening in human cells using a fluorinated DNA target. A fluorinated probe is chemically linked to the target DNA to allow monitoring the intracellular interaction with ligands. The highly sensitive ¹⁹F chemical shift allows separating different ligand-bound and unbound species.

43. Li C, Wang G-F, Wang Y, Creager-Allen R, Lutz EA, Scronce H, Slade KM, Ruf RAS, Mehl RA, Pielak GJ: **Protein (¹⁹F) NMR in Escherichia coli.** *J Am Chem Soc* 2010, **132**:321–327.

44. Bao H-L, Ishizuka T, Sakamoto T, Fujimoto K, Uechi T, Kenmochi N, Xu Y: **Characterization of human telomere RNA G-quadruplex structures in vitro and in living cells using ¹⁹F NMR spectroscopy.** *Nucleic Acids Res* 2017, **45**:5501–5511.

45. Airoldi C, Giovannardi S, La Ferla B, Jiménez-Barbero J, Nicotra F: **Saturation Transfer Difference NMR Experiments of Membrane Proteins in Living Cells under HR-MAS Conditions: The Interaction of the SGLT1 Co-transporter with Its Ligands.** *Chemistry – A European Journal* 2011, **17**:13395–13399.

46. Claasen B, Axmann M, Meinecke R, Meyer B: **Direct Observation of Ligand Binding to Membrane Proteins in Living Cells by a Saturation Transfer Double Difference (STDD) NMR Spectroscopy Method Shows a Significantly Higher Affinity of Integrin α IIb β 3 in Native Platelets than in Liposomes.** *J Am Chem Soc* 2005, **127**:916–919.

47. Bouvier G, Simenel C, Jang J, Kalia NP, Choi I, Nilges M, Pethe K, Izadi-Pruneyre N: **Target Engagement and Binding Mode of an Antituberculosis Drug to Its Bacterial Target Deciphered in Whole Living Cells by NMR.** *Biochemistry* 2019, **58**:526–533.

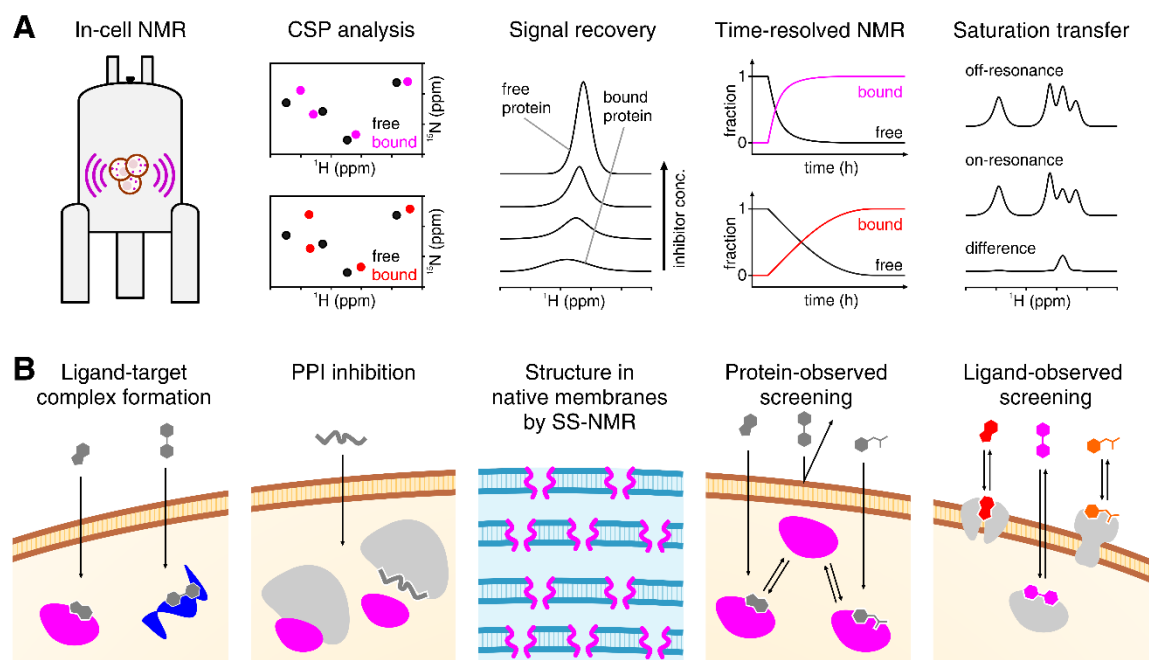
48. Mari S, Invernizzi C, Spitaleri A, Alberici L, Ghitti M, Bordignon C, Traversari C, Rizzardi G-P, Musco G: **2D TR-NOESY Experiments Interrogate and Rank Ligand–Receptor Interactions in Living Human Cancer Cells.** *Angewandte Chemie International Edition* 2010, **49**:1071–1074.

49. Potenza D, Vasile F, Belvisi L, Civera M, Araldi EMV: **STD and trNOESY NMR Study of Receptor–Ligand Interactions in Living Cancer Cells.** *ChemBioChem* 2011, **12**:695–699.

50. Primikyri A, Sayyad N, Quilici G, Vrettos EI, Lim K, Chi S-W, Musco G, Gerothanassis IP, Tzakos AG: **Probing the interaction of a quercetin bioconjugate with Bcl-2 in living human cancer cells with in-cell NMR spectroscopy.** *FEBS Letters* 2018, **592**:3367–3379.
51. Luchinat E, Barbieri L, Cremonini M, Banci L: **Protein in-cell NMR spectroscopy at 1.2 GHz.** *J Biomol NMR* 2021, **75**:97–107.
52. Burz DS, Breindel L, Shekhtman A: **Improved sensitivity and resolution of in-cell NMR spectra.** *Methods Enzymol* 2019, **621**:305–328.
53. Barbieri L, Luchinat E: **Monitoring Protein-Ligand Interactions in Human Cells by Real-Time Quantitative In-Cell NMR using a High Cell Density Bioreactor.** *JoVE (Journal of Visualized Experiments)* 2021, doi:10.3791/62323.
54. Boeszoermyeni A, Chhabra S, Dubey A, Radeva DL, Burdzhiev NT, Chanev CD, Petrov OI, Gelev VM, Zhang M, Anklin C, et al.: **Aromatic 19F-13C TROSY: a background-free approach to probe biomolecular structure, function, and dynamics.** *Nat Methods* 2019, **16**:333–340.
55. Orton HW, Qianzhu H, Abdelkader EH, Habel EI, Tan YJ, Frkic RL, Jackson CJ, Huber T, Otting G: **Through-Space Scalar 19F-19F Couplings between Fluorinated Noncanonical Amino Acids for the Detection of Specific Contacts in Proteins.** *J Am Chem Soc* 2021, **143**:19587–19598.

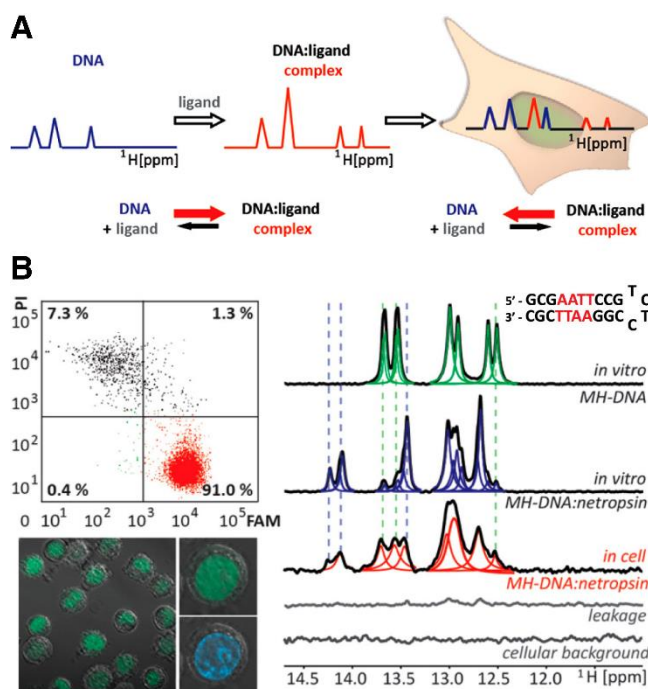
Figures

Figure 1



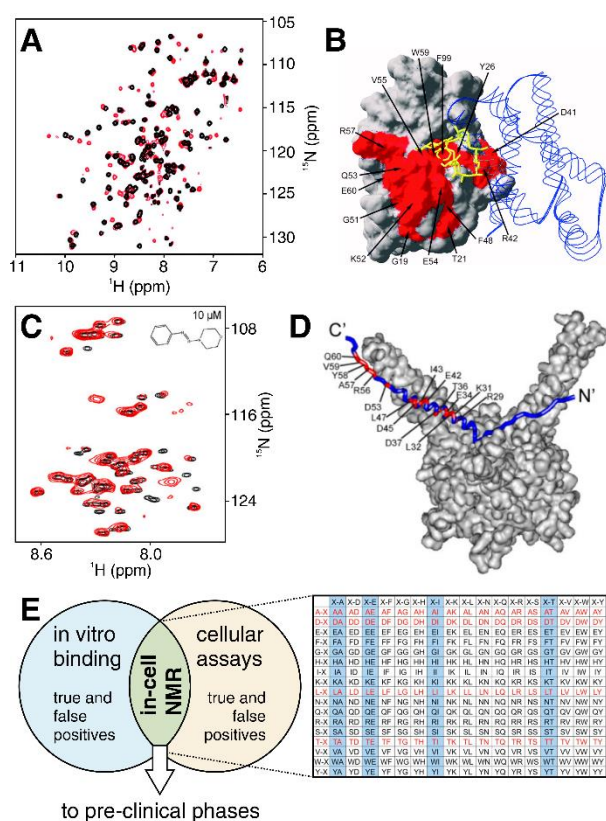
Overview of in-cell NMR approaches for characterizing ligand-target complexes and drug screening. (A) NMR methods, from left to right: intact living cells are analyzed by high-field NMR; CSP induced by ligand binding allows mapping the interaction surface and structural rearrangements; Recovery of signal intensity can indicate the dissociation of a protein from a slow-tumbling complex; Time-resolved NMR over several hours reveals binding kinetics; Saturation transfer-based experiments can probe the interaction of ligands with intact cells. (B) Applications, from left to right: treating cells with ligands allows studying the interaction with an intracellular protein (magenta) or nucleic acid (blue); inhibitors of protein-protein interactions (PPI) can be screened by relying on the increase of signal from a labeled protein released from the complex with a partner; the structure of membrane-bound drugs is investigated in cryogenically-cooled native membranes by DNP-assisted SS-NMR; protein-observed drug screening gives insights on the kinetics of membrane diffusion and intracellular binding affinity; ligand-observed screening identifies ligands interacting with membrane-bound or intracellular targets.

Figure 2



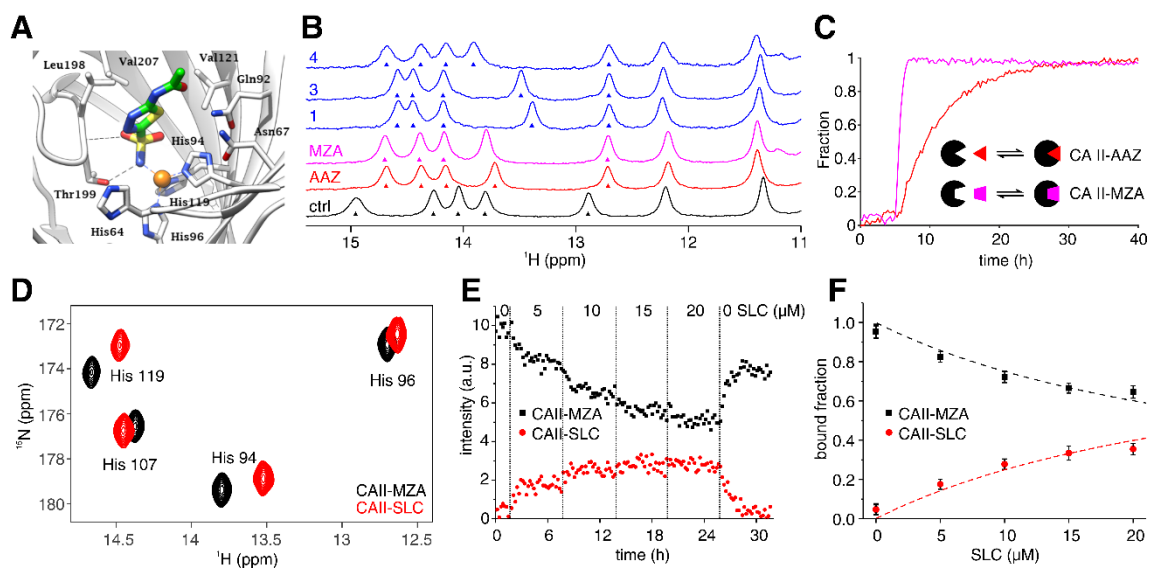
DNA-ligand complexes observed in human cells by NMR. (A) Strategy for in-cell NMR sample preparation: the complex between a short DNA motif and a ligand is preformed and analyzed *in vitro*, and subsequently introduced in human cells for in-cell NMR analysis; (B) Internalization efficiency of a 24-mer DNA hairpin (MH-DNA) and cell viability are assessed by flow cytometry (top left), nuclear localization is confirmed by confocal microscopy (bottom left), while the stability of the intracellular complex is evaluated by chemical shift analysis (right). Reproduced with permission from Krafcikova et al. [25] Copyright 2019 American Chemical Society.

Figure 3



Protein-observed drug screening against protein-protein interactions in bacteria. (A) Drug binding induces CSP in the 2D in-cell NMR spectra of a target protein; (B) Protein surface residues involved in the interaction with the drug (red), which interferes with the complex formation with a cellular partner (blue); (C) Drug binding causes release of the unfolded protein Pup from its complex with Mpa, leading to signal increase; (D) Model of the interaction between Pup (blue) and Mpa (gray), where residues perturbed by the drug are shown (red); (E) Rationale for drug screening by in-cell NMR, combining advantages from in vitro binding studies and cellular assays (left); the screening throughput is increased by matrix approaches (right). Reproduced with permission from Xie et al. [16] Copyright 2009 American Chemical Society (panels A, B, E right) and from DeMott et al. [35] Copyright 2018 American Chemical Society (panels C, D, E left).

Figure 4



Drug screening, binding kinetics and thermodynamics in human cells. (A) 3D view of a drug (acetazolamide, AAZ) bound to the active site of human CA II (PDB: 3HS4); (B) In-cell NMR spectra showing CA II in the absence of ligands (black) and bound to AAZ (red), methazolamide (MZA, magenta), and other ligands (blue); (C) Intracellular drug binding monitored by time-resolved NMR reveals different membrane diffusion kinetics for AAZ (red) and MZA (magenta); (D) 2D NMR spectra of CA II bound to a reference ligand (MZA, black) and to a test ligand (SLC, red); (E) Time-resolved concentration profiles of the two adducts at increasing concentration of test ligand and constant reference ligand; (F) Bound fractions obtained at the equilibrium for each step are fitted to obtain the affinity constant of the test ligand. Reproduced with permission from Luchinat et al. [18] Copyright 2020 Luchinat et al. (panels A, B), from Luchinat et al. [36] Copyright 2020 American Chemical Society (panel C), and from Luchinat et al. [38] under the terms of the CC-BY 4.0 license (panels D-F).