Journal of Magnetic Resonance 306 (2019) 202–212

Contents lists available at ScienceDirect

Journal of Magnetic Resonance

journal homepage: www.elsevier.com/locate/jmr

Cells, drugs and NMR

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ARTICLE INFO

Article history:
Received 18 May 2019
Revised 8 June 2019
Accepted 8 July 2019
Available online 13 July 2019

Keywords:
In-cell NMR
In vivo NMR
On-cell NMR
Whole-cell NMR
Metabolomics
Solid-state NMR
Dynamic nuclear polarization

ABSTRACT

Nuclear magnetic resonance (NMR) spectroscopy is a versatile tool for investigating cellular structures and their compositions. While *in vivo* and whole-cell NMR have a long tradition in cell-based approaches, high-resolution in-cell NMR spectroscopy is a new addition to these methods. In recent years, technological advancements in multiple areas provided converging benefits for cellular MR applications, especially in terms of robustness, reproducibility and physiological relevance. Here, we review the use of cellular NMR methods for drug discovery purposes in academia and industry. Specifically, we discuss how developments in NMR technologies such as miniaturized bioreactors and flow-probe perfusion systems have helped to consolidate NMR's role in cell-based drug discovery efforts.

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1. Introduction

NMR spectroscopy has been an integral part of drug discovery pipelines for many years [1] with important roles in structure- and fragment-based discovery efforts [2,3], structure-activity relationship (SAR) assessments [4], metabolomics [5] and as an essential analytical tool at different stages of drug development [6]. In these applications, however, NMR is used in a reductionist manner on simplified, homogenous systems of sometimes little biological relevance. Cells, on the other hand, are equally important tools in drug discovery as they enable the testing of biological compound activities in simple physiological settings. Cell-based assays reveal whether molecules cross cell membranes (permeability), reach their cellular targets and elicit the desired responses (activity) without detrimental effects on cell viability (toxicity). Accordingly, cell-based assays represent key decision points in all stages of drug development. On the other hand, these assays do not usually reveal whether, and how, compounds interact with their intended biological targets. For these reasons, the prospects of merging NMR methods with applications aimed at understanding cellular drug activities are highly attractive. Here, we discuss how in-cell NMR spectroscopy can harness the combined benefits of NMR and cell-based assays for drug discovery purposes. We further provide an outlook towards developments that, we believe, will strengthen the usefulness of in-cell NMR measurements for the broader drug discovery community.

From a drug discovery perspective, in-cell NMR can provide valuable information on the binding of a compound to its target, a concept known as cellular target engagement. Whenever estimates of the binding affinity within a cell can be made, such data may help to clarify differences in apparent efficacies between biochemical and cell-based assays, known as cellular shift. This may happen when compounds interact with targets in an ATP competitive manner, for example. Where high quality NMR spectra of intracellular targets can be acquired, it will be possible to determine whether structural models of purified components are relevant in cellular milieus. Lastly, and perhaps most excitingly, information about metabolic changes that occur in response to drug administration and the kinetics of cellular drug activities become experimentally tractable. Clearly, such in-cell NMR approaches will prove most valuable in the mid to late stages of drug development processes, when optimized compound-to-target binding affinities are in the sub-micromolar range i.e. in slow-exchange on the NMR timescale.

In 2001, Serber and Dötsch defined the modern concept of in-cell NMR spectroscopy [7], which was inspired by earlier '*in vivo* NMR' work aimed at detecting NMR-active ^{1}H, ^{31}P and ^{13}C metabolite nuclei at natural-abundance in intact cells [8]. By contrast, in-cell NMR was developed to study larger biomolecular structures such as proteins, nucleic acids and proteoglycans that are specifically enriched with NMR-active ^{15}N, ^{19}F, (^{2}H) and/or ^{13}C isotopes, which are then exploited to selectively detect these biomolecules

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https://doi.org/10.1016/j.jmr.2019.07.018
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against the backdrop of all other unlabeled, cellular components. Therefore, in-cell NMR approaches require dedicated procedures to enrich or deliver NMR-detectable targets into cells (see below). As recently pointed out by Lippens and colleagues, the boundary between *in vivo* and in-cell NMR applications is fluid and many cellular NMR approaches combine elements of both methods [9]. For the sake of this review, we will further extend the definition of in-cell NMR spectroscopy to also include applications that involve extracellular structures such as membranes and membrane proteins, often referred to as on-cell or whole-cell NMR, [10,11].

In-cell solution-state NMR spectroscopy was originally devised to study overexpressed and isotope-labeled proteins in bacteria [7]. As we learned over the years, this may have been an unfortunate first choice. While early leakage problems tainted the reputation of the method [12], it was soon realized that the bacterial cytosol is more crowded (150–300 mg/mL total macromolecular content) than other cellular milieus, especially those of higher eukaryotic organisms such as *Xenopus laevis* oocytes and mammalian cells (50–250 mg/mL) [13]. Macromolecular crowding contributes to the higher 'apparent' viscosity of intracellular environments, which increases rotational correlation times (τ_c) of NMR-observable biomolecules and enhances longitudinal (T1) and transverse (T2) relaxation, all of which impact NMR signals in a negative way. In addition, background molecules in crowded cytosols often interact weakly and transiently with intracellular NMR targets [14,15], which causes signal attenuation and concomitant line-broadening through quinary structure effects [16]. Correspondingly, highly crowded intracellular environments such as those of bacteria result in poorer spectral NMR characteristics than dilute ones, especially for folded, globular proteins [17]. For these reasons, in-cell NMR experiments in bacteria are particularly challenging [18]. It is important to note that even mammalian cells exhibit varying contents of nucleic acids and proteins, which complicates conclusions regarding their general suitability for in-cell NMR measurements [19]. While quinary structure interactions and intracellular viscosity are disadvantageous for in-cell NMR measurements of folded and disordered proteins [20–24], their combined effects are less detrimental for the latter [25,26]. Therefore, disordered proteins are often considered superior in-cell NMR targets [18]. Despite their importance in human disease processes ranging from different forms of cancers to neurodegenerative disorders, disordered proteins had been deemed undruggable for a long time. This notion is being actively revised and several companies are currently pursuing drug discovery programs on disordered targets [27–29].

Irrespective of the shortcomings of early in-cell NMR systems, the method persevered and evolved from prokaryotic to eukaryotic cells, including *Xenopus laevis* oocytes [30], yeast [31], insect [32] and cultured mammalian cells [33–36]. Sample preparation routines expanded from protein overexpression and in-cell NMR measurements in the same cell types (bacteria, insect and mammalian cells) to delivery by microinjection [30], cell-penetrating peptides [33], pore-forming toxins [34] and transduction via electroporation [36,37]. These procedures greatly expanded the scope of in-cell NMR applications and the range of cells amenable to these experiments [38]. In-cell NMR of DNA and RNA was successfully introduced [39] and the method was adapted for solid-state NMR applications [40]. By now, both types of approaches are well-established [41,42]. In-cell NMR featured prominently in several landmark studies [33,35,36,43–51] and stimulated the development of other spectroscopic in-cell applications including those by electron paramagnetic resonance (EPR) [52,53] and single-molecule Förster resonance energy transfer (smFRET) spectroscopies [54].

In principle, in-cell NMR offers several attractive features for drug discovery [55]. The ability to monitor interactions of unlabeled small-molecule compounds with labeled cellular targets at atomic resolution in live cells delineates many of the key parameters of cell-based assays. By switching isotope labels from targets to compounds, the cellular fate of these molecules can be studied in a reciprocal manner. In parallel, cell states can be assessed from metabolite analyses, or via reporter readouts (see below). These tantalizing prospects were emphasized in the very first in-cell NMR publication in 2001 [7] and reiterated in almost every in-cell NMR review since then [38,56,57]. Probably the truly first drug-related cellular NMR study was performed about two decades earlier by Jacob and Schaefer, then at Monsanto [58]. In 1982, they grew *Aerococcus viridans* in L-[ε^{15}N] lysine-supplemented growth medium to produce specific ^{15}N-isotope containing cross-links in their outer peptidoglycan structures. In turn, Jacob and Schaefer used intact cells to detect amide (^{15}NH) and amine ($^{15}NH_2$) resonances resulting from these cross-links by cross-polarization (CP) magic-angle spinning (MAS) solid-state NMR spectroscopy via direct ^{15}N-detection. Next, they grew cells in the presence of penicillin, the classical antibiotic known to inhibit the bacterial transpeptidase that catalyzes the formation of peptidoglycan cross-links and repeated their experiments on these cells. As expected, penicillin-treated bacteria revealed reduced cross-link NMR signals, which unambiguously confirmed the mode of antibiotic action [58]. By characterizing the binding of an exogenously added, small-molecule compound to intracellularly expressed and isotope-labeled CheY in live bacteria, Hubbard and Fosberry at GlaxoSmithKline reported the first 'modern', drug-related in-cell NMR analysis [59]. While their paper stimulated several follow-up studies (see Table 1 for an overview), later efforts remained academic with one notable exception [60]. Possible reasons for the reluctance on the industry side to engage in these approaches may relate to the considerable variabilities, poor reproducibility and ill-defined metabolic consistencies of early in-cell NMR samples [61,62]. Today, many of these issues have been resolved, also due to the development of miniaturized bioreactors and flow-probes that maintain cells under controlled metabolic conditions inside NMR spectrometers, thus offering major stability breakthroughs (see chapter 6 for details) [62–69].

In general, in-cell NMR applications for drug discovery can be divided into five types depending on which part carries the NMR-observable isotope(s). This can be the actual drug target, either a protein, nucleic acid or other biomolecule (Fig. 1A), the drug compound (Fig. 1B), peptide- or metabolite-based 'reporters' that convey target-drug interactions (Fig. 1C), specific metabolites that reveal cellular conditions after drug applications (Fig. 1D), or entire cell structures such as membranes and their components, against which drugs are raised (Fig. 1E). In the following chapters, we outline examples of all five types of applications.

2. Target-detected in-cell NMR

The first in-cell NMR experiments of target-drug interactions were performed on isotope-labeled proteins and unlabeled small molecules that were added to the respective in-cell NMR samples (Fig. 1A). One interesting example of this target-detected approach is that of the peptidyl-prolyl cis-trans isomerase FKBP12, which binds to the major immunosuppressants FK506 and rapamycin [33,70]. In bacteria, overexpressed FKBP12 was not detected due to transient quinary interactions with cellular components [70]. By contrast, in-cell NMR spectra of free FKBP12 in human HeLa cells were of good quality and allowed Shirakawa and colleagues to delineate high-resolution insights into the binding of FK506 and rapamycin to intracellular FKBP12 [33]. The authors found that these target-drug complexes closely resembled individual *in vitro* interactions, which led them to conclude that both compounds

crossed the mammalian plasma membrane, reached the cytoplasm in intact forms and produced specific FKBP12 complexes without major off-target effects, based on applied drug and intracellular target concentrations. In bacteria, Shekhtman realized that sequential overexpression of FKBP12 and FRB, the FKBP binding domain of the mammalian target of rapamycin (mTOR) kinase, yielded well-detectable in-cell NMR signals of the assembled complex [70]. The rationale to sequentially overexpress two (or more) interacting proteins from different plasmids for studying intracellular protein-protein interactions by in-cell NMR had been reported earlier by

Table 1
Overview of drug-related cellular NMR studies. (See below-mentioned references for further information.)

	NMR detect										
Year	Target	Drug	Reporter	Metabolite	Structure	Origin	Cell Type	Target Type	Compound/ Drug	Isotope	NMR-type & [Reference]
2019	●					human	HeLa	hairpin DNA mismatch DNA	netropsin naphtalenophanes	^{1}H	solution NMR Trantirek *et al.* 2019 **[72]**
		●				human	HEK293T HeLa	Stat3 mRNA	anti-sense oligonucleotide	^{1}H ^{31}P	solution & ssNMR Petzold *et al.* 2019 **[73]**
				●		human	HeLa	ATP/ADP metabolites	calix-NH2 5-fluorouracil (5-FU)	^{31}P	solution NMR **bioreactor** Fonseca & Cruz *et al.* 2019 **[64]**
					●	bacteria	*Escherichia coli*	phospholipid headgroups	ampicillin maculatin1.1	^{31}P	solid-state NMR Separovic & Sani *et al.* 2019 **[125]**
2018		●				bacteria	*Mycobacterium smegmatis*	cytochrome *b* protein	imidazo-pyrimidine amide (IPA317)	^{1}H	solution NMR Izadi-Pruneyre *et al.* 2018 **[85]**
	●			●		human	HEK293T	glucose/lactate ATP/ADP superoxide dismutase (SOD1) protein	no compound/drug testing	^{1}H ^{31}P ^{15}N	solution NMR **bioreactor** Luchinat & Ravera *et al.* 2018 **[65]**
		●				human	Jurkat	Bcl-2 protein	quercetin-alanine inhibitor HA 14-1	^{1}H	solution NMR Tzakos *et al.* 2018 **[83]**
		●				bacteria	*Micrococcus flavus*	cell wall precursor lipid II	antimicrobial peptide nisin lantibiotic	^{1}H ^{13}C ^{15}N	solid-state NMR & DNP Weingarth *et al.* 2018 **[86]**
	●					bacteria	*Escherichia coli*	prokaryotic ubiquitin-like protein (Pup) mycobacterial proteasome ATPase (Mpa) complex	NIH/NCI Diversity Set III PPI inhibitors	^{1}H ^{15}N	solution NMR STINT NMR Shekhtman *et al.* 2018 **[138]**
	●		●	●		human	HeLa S3 (non adherent)	Thioredoxin (Trx) protein GSH/GSSG glutathione	Trx reductase inhibitor aurothioglucose (ATG)	^{1}H ^{13}C	solution NMR **bioreactor** Nishida & Shimada *et al.* 2018 **[99]**
	●					human	HeLa	ATP/ADP Trx protein	tetracycline streptomycin chloramphenicol antibiotics	^{1}H ^{31}P ^{15}N	solution NMR **bioreactor** Shekhtman *et al.* 2018 **[130]**
				●		yeast	*Saccharomyces cerevisiae*	pyruvate metabolites	no compound/drug testing	^{1}H ^{13}C	solution NMR & D-DNP Hilty *et al.* 2018 **[103]**
2017	●					human	HeLa	human adenylate kinase 1 (hAK1) protein	no compound/drug testing	^{1}H ^{15}N	solution NMR **bioreactor** Ito & Kigawa *et al.* 2017 **[67]**
		●				human	MDA-MB-231	$\alpha 1\beta 5$ integrin protein	RGD peptidomimetics	^{1}H	solution NMR Belvisi & Potenza *et al.* 2017 **[77]**
2016					●	bacteria	*Escherichia coli Bacillus subtilis*	cell wall	caerin 1.1 aurein 1.2 antimicrobial peptides	^{2}H	solid-state NMR Separovic & Marcotte *et al.* 2016 **[124]**
				●	●	human	HeLa H460	cellular lipids	lactate dehydrogenase (LDH) inhibitor oxamate	^{2}H ^{13}C	solid-state NMR Schaefer & Patti *et al.* 2016 **[105]**
	●					bacteria	*Escherichia coli*	Bcl-XL - Bak protein-peptide complex	no compound/drug testing	^{1}H ^{13}C	solution NMR & targeted DNP Etzkorn *et al.* 2016 **[135]**
			●	●		human	U87 glioblastoma	GSH/GSSG glutathione	temolozomide (TMZ)	^{1}H ^{13}C	solution NMR Park *et al.* 2016 **[100]**
2015	●					amphibian	*Xenopus laevis*	G-quadruplex DNA	2,6-N,N'-(methyl-quinolin-3-yl)-pyridine dicarboxamide (360A)	^{1}H ^{13}C ^{15}N	solution NMR Mergny *et al.* 2015 **[71]**
			●			amphibian human	*Xenopus laevis* K562 (lysates)	Kinase Activity Reporters (KARs) peptides	various kinase inhibitors	^{1}H ^{15}N	solution NMR Selenko *et al.* 2015 **[92]**
			●			human	HepG2 NKNT-3	glucose-derived metabolites	lactate dehydrogenase (LDH) inhibitor galloflavin	^{1}H ^{13}C	solution NMR Park *et al.* 2015 **[106]**
	●					bacteria	*Escherichia coli*	prokaryotic ubiquitin-like protein (Pup)	peptide aptamers	^{1}H ^{15}N	solution NMR Shekhtman *et al.* 2015 **[139]**

Table 1 (*continued*)

Year	NMR detect: Target	Drug	Reporter	Metabolite	Structure	Origin	Cell Type	Target Type	Compound/ Drug	Isotope	NMR-type & [Reference]
2014		●				bacteria	*Escherichia coli*	cefotaxime ceftazidime tigacycline aztreonam imipenem meropenem antibiotics	metallo-β-lactamase subclass 1 (NDM-1) inhibitors L-captopril & EDTA	^{1}H	solution NMR Breeze & Hu *et al.* 2014 [**60**]
			●			human	HEK293T	fatty acid amide hydrolase (FAAH) fluorinated substrate analogue (ARN1203)	FAAH inhibitor URB597 new inhibitor compounds	^{19}F	solution NMR Garau & Dalvit *et al.* 2014 [**96**]
2013			●			human	10 patient-derived colon cancer cell lines (lysates)	Erk kinase reporter peptide	U0126	^{1}H ^{15}N	solution NMR Selenko *et al.* 2013 [**93**]
	●		●			human	red blood cells	human carbonic anhydrase I (hCAI)	azetazolamide (AAZ) benzensulfonamide (BS)	^{19}F	solution NMR Hamachi *et al.* 2013 [**94**]
2012			●			amphibian	*Xenopus laevis*	PEPT1 & PAT1 transporters sarcosine glycyl-sarcosine (Gly-Sar) cargo reporters	no compound/drug testing	^{1}H	solution NMR Kremer & Stolz *et al.* 2012 [**134**]
2011	●					bacteria	*Escherichia coli*	Atox 1 protein	cisplatin	^{1}H ^{15}N	solution NMR Banci & Bertini *et al.* 2011 [**140**]
			●			human	HeLa (nuclear extracts)	histone H4 peptides	no compound/drug testing	^{1}H ^{15}N	solution NMR Schwarzer *et al.* 2011 [**90**]
		●				human	ECV304	αvβ3 integrin protein	RGD peptidomimetics	^{1}H	solution NMR Potenza *et al.* 2011 [**76**]
2010		●				human	MR300 MSR3	αvβ3 integrin aminopeptidase N (CD13)	RGD peptidomimetics	^{1}H	solution NMR Musco *et al.* 2010 [**78**]
2009	●					bacteria	*Escherichia coli*	FKBP12 - FRB protein complex	rapamycin ascomycin di-peptide inhibitors	^{1}H ^{15}N	solution NMR Shekhtman *et al.* 2009 [**70**]
	●					human	HeLa	FKBP12 protein	rapamycin FK504	^{1}H ^{15}N	solution NMR Shirakawa *et al.* 2009 [**33**]
2008		●				human	platelets	αIIbβ2 integrin protein	RGD peptidomimetics	^{1}H	solution NMR Potenza *et al.* 2008 [**75**]
2007	●				●	bacteria	*Escherichia coli*	polysialic acid (PSA)	no compound/drug testing	^{1}H ^{13}C ^{15}N	solution NMR Freedberg *et al.* 2007 [**133**]
2006				●		bacteria	*Staphylococcus aureus*	cell wall peptidoglycans	vancomycin eremomycin chloroeremomycin	^{1}H ^{13}C ^{15}N ^{19}F	solid-state NMR Schaefer *et al.* 2006 [**136**]
				●		bacteria	*Staphylococcus aureus*	cell wall peptidoglycans	oritavancin analogue	^{1}H ^{13}C ^{15}N ^{19}F	solid-state NMR Schaefer *et al.* 2006 [**137**]
2005		●				human	platelets	αIIbβ3 integrin protein	RGD peptidomimetics	^{1}H	solution NMR Meyer *et al.* 2005 [**74**]
2004	●					bacteria	*Escherichia coli*	calmodulin NmrA FKBP12 proteins	phenoxybenzamine hydrochloride	^{1}H ^{13}C ^{15}N	solution NMR Doetsch *et al.* 2004 [**141**]
2003	●					bacteria	*Escherichia coli*	CheY protein	BRL-16492PA	^{1}H ^{15}N	solution NMR Hubbard *et al.* 2003 [**59**]
1983					●	bacteria	*Aerococcus viridans*	cell wall peptidoglycans	penicillin	^{15}N	solid-state NMR Jacob & Schaefer *et al.* 1983 [**58**]

the same group [43]. It proved essential for further experiments aimed at identifying compounds that disrupted the FKBP12-FBR complex [70]. In a first step, Shekhtman and colleagues added rapamycin to their in-cell NMR samples, which turned the low-affinity FKBP12-FBR interaction into the mature, high-affinity FKBP12-FBR-rapamycin complex. A related compound, ascomycin, was then added to selectively displace rapamycin from the ternary assembly to form the respective FKBP12-FBR-ascomycin complex. Thus, the competitive differences between rapamycin and ascomycin binding to cellular FKBP12-FBR were clearly revealed by in-cell NMR readouts. Next, they screened a library of dipeptides for their ability to disrupt the FKBP12-FBR complex and they used rapamycin/ascomycin to test whether peptides can be competed off with these drugs [70]. They identified several FKBP12-FBR inhibitors

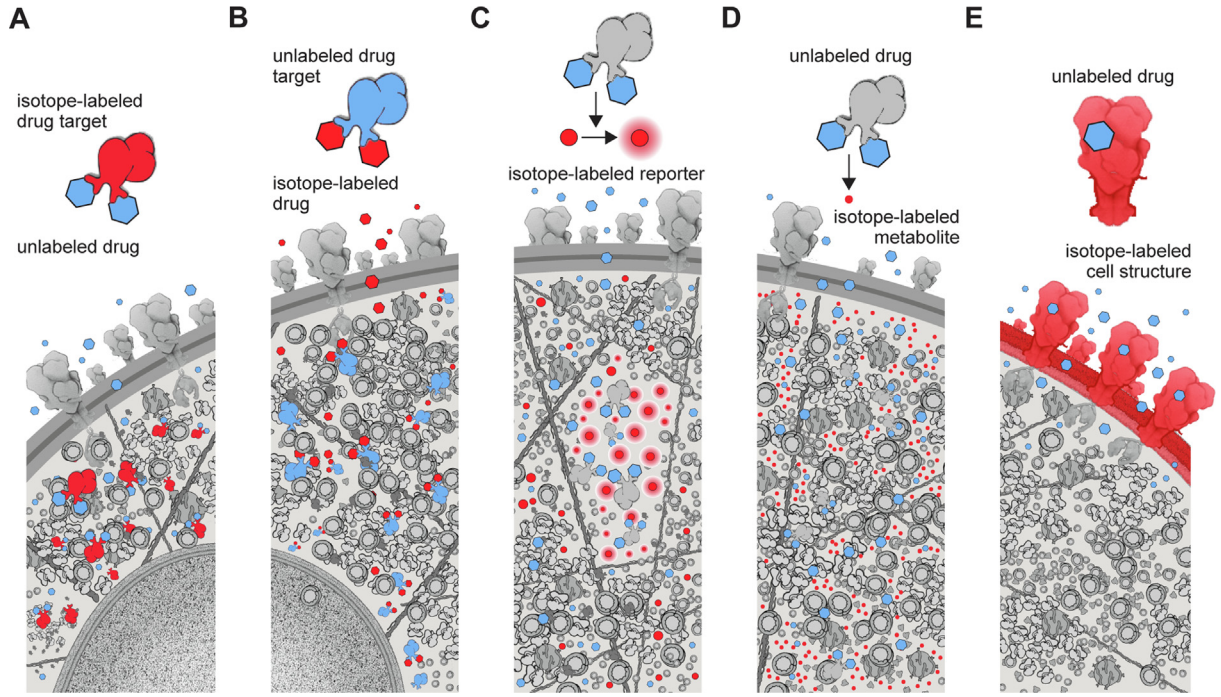

Fig. 1. Schematic overview of drug-related cellular NMR approaches. (A) Isotope-labeled drug targets including proteins and nucleic acids are specifically enriched in the cytoplasm of investigated cells, shown here schematically for a divalent target protein (red) interacting with two molecules of a hypothetical drug compound (blue). (B) Inverse scenario in which the drug compound represents the isotope-labeled species (red) binding to the same, non-labeled target (blue). (C) Target-compound interactions may be indirectly conveyed by an isotope-labeled reporter entity (red) whose spectral properties change upon intracellular drug binding (blue). (D) Cellular effects of drug treatment monitored via spectral changes of isotope-labeled metabolites (red). (E) Isotope-enrichment of entire cellular structures such as membranes and integral components enables whole-cell NMR studies of drug interactions (blue). The cellular landscape drawing was created in CellPAINT [142].

with non-overlapping binding sites for rapamycin or ascomycin, which may be further developed into new classes of immunosuppressant drugs.

Recent in-cell NMR drug interaction studies introduced DNA as a compound target [41]. In 2015, Mergny and colleagues reported binding of a G-quadruplex-specific drug to isotope-labeled d $(TG_4T)_4$ in *Xenopus laevis* oocytes [71], whereas the group of Trantirek performed similar in-cell NMR experiments on DNA in the nuclei of intact mammalian cells [72]. Petzold and colleagues extended this concept to investigate the interaction of AstraZeneca's Danvatirsen, an antisense oligo-nucleotide currently in clinical trials against various cancers, with its STAT3 mRNA target [73]. In this approach, however, NMR-detection was based on the compound rather than the target, which provides the transition for the next chapter.

3. Compound-detected in-cell NMR

NMR approaches to monitor the binding of small molecules to native cellular structures (Fig. 1B) were originally based on the detection of compound protons (^{1}H) via saturation transfer difference (STD), saturation transfer double difference (STDD), and transferred NOE (trNOE) experiments [2]. Interactions probed with these methods include binding of small molecules and peptides to cell surface proteins such as integrins [74–79] and G-protein coupled receptors (GPCRs) [80], two important classes of drug targets [81,82]. Compound interactions with intracellular proteins were similarly studied with STD and trNOE experiments, as exemplified by efforts to interrogate the binding of a quercetin-alanine bioconjugate to the apoptosis inhibitor Bcl-2 [83], another drug target of significant interest [84]. This year, Izadi-Pruneyre and Nilges used whole-cell STD measurements and computational docking, to determine the binding mode of an anti-tuberculosis imidazopyridine amide (IPA) drug to intracellular cytochrome *b* in its native membrane environment in live bacteria [85]. While these approaches (mostly) exploited natural abundance compound-proton NMR signals, and did not require site-selective isotope incorporation, a recent study by Breunkink and Weingarth reported the interaction of uniformly [^{13}C,^{15}N]-isotope labeled nisin, an antimicrobial peptide, with the native lipid II cell wall precursor of intact *Micrococcus flavus* membranes [86]. Lantibiotics that specifically target the lipid II system and perforate bacterial cell walls including nisin and vancomycin, represent essential tools to fight multi drug-resistant pathogens and, as such, are of great pharmaceutical importance [87]. Structural information about how these compounds interact with lipid II on bacterial membranes was previously not available. Using dynamic nuclear polarization (DNP)-enhanced solid-state NMR spectroscopy, Breunkink and Weingarth showed that that nisin bound native lipid II in a conformation that was different from its structure in reconstituted liposomes and organic solvents [88]. Thereby, they provided a first glimpse towards the great potential of cellular NMR applications to delineate native binding modes for structure-based drug discovery efforts.

4. Reporter-detected in-cell NMR

Another application of in-cell NMR entails the use of reporters to convey target-compound interactions in an indirect manner (Fig. 1C). Given that many drug targets are enzymes, with compounds designed to modulate their activities, NMR-active reporter substrates can be engineered to read out the effects of enzyme-compound interactions. As such, they monitor levels of inhibition or stimulation in native, cellular environments. By analyzing

reporter turnover with time-resolved NMR spectroscopy, modulatory in-cell activities can be deduced [89]. One advantage of this approach is that experimental limitations regarding enzyme/target sizes and compound chemistries are avoided because reporters can be based on synthetic peptides or minimal substrate analogues into which NMR-observable isotopes are incorporated at chosen positions. We introduced several such reporters in the past, including engineered histone tail peptides for monitoring acetyltransferase and deacetylase activities [90,91], or optimized consensus substrates to measure cellular kinase and phosphatase activities [92] and drug-induced kinase inhibition in cancer patient-derived cell lysates [93]. In 2013, Shirakawa and Hamachi extended this reporter concept to identify competitive inhibitors against human carbonic anhydrase I (hCAI) [94], a broad-spectrum drug target with modulating compounds in clinical use since the 1950s [95]. Specifically, they used an intracellular ligation approach based on ligand-directed tosyl (LDT) chemistry to tether a ^{19}F fluorine reporter onto endogenous hCAI in red blood cells. The trivalent reporter contained a fluorine moiety for NMR detection, a reactive sulfoxide group for coupling and a generic hCAI ligand for targeting the compound to the active site of the enzyme. In its tethered form, the single ^{19}F NMR signal reported on the ligand-bound state of hCAI. By titrating the competitive hCAI inhibitors acetazolamide (AAZ) and benzenesulfonamide (BS) to their cells, displacement of the generic reporter ligand resulted in chemical shift changes of the ^{19}F resonance signal. By measuring these chemical shifts, the authors determined relative drug binding affinities and respective ligand-drug exchange rates in a native cellular setting [94]. Another application of ^{19}F fluorine-based in-cell NMR reporters for drug discovery was described by Garau and Dalvit [96]. In their study, they over-expressed fatty acid amid hydrolase (FAAH) in human HEK293 cells and used cell-membrane preparations to screen for drugs that modulated FAAH activity [97]. Employing a ^{19}F-fluorinated FAAH substrate analogue and time-resolved NMR spectroscopy, they referenced enzyme inhibition against the commercial antagonist URB597 and successfully screened a compound library for new FAAH inhibitors. Given the prominence of ^{19}F NMR in general drug discovery programs [98] and its suitability for cell-based applications [17], we feel that the prospects for drug-related fluorine-NMR approaches in cells are particularly encouraging and await more comprehensive explorations in the future. An intriguingly different reporter approach was recently introduced by Nishida and Shimada [99]. Following a similar dual target-reporter idea, they monitored the oxidation state of selectively alanine-[$^{13}CH_3$] labeled thioredoxin (Trx) in human HeLa cells inside a flow-probe bioreactor (see chapter 6 for details). In parallel, they measured intracellular levels of reduced (GSH) and oxidized (GSSG) glutathione acting as a general in-cell reporter of the cytoplasmic redox state (GSH/GSSG ratio). While they delivered ^{13}C-labeled Trx via a pore-forming toxin [34], they accumulated intracellular ^{13}C-GSH via metabolic scrambling from exogenously added, uniform ^{13}C-isotope labeled cysteine. This elegant reaction and the highly useful NMR redox reporter that resulted from it had been described earlier by Park and colleagues, who tested whether the anti-cancer drug temozolomide (TMZ) induced increased levels of reactive oxygen species (ROS) in TMZ-resistant U87 glioblastoma cells [100]. Indeed, they found that ROS and corresponding GSSG levels were significantly elevated in TMZ-resistant versus non-resistant tumor cells. In the dual target-reporter study by Nishida and Shimada, simultaneous in-cell detection of ^{13}C resonances of reduced and oxidized Trx, and GSH/GSSG, allowed them to correlate cytoplasmic redox states to the oxidation behavior of intracellular Trx. One goal of their work was to evaluate whether the cellular activity of the Trx reductase inhibitor aurothioglucose (ATG), a drug used for the treatment of rheumatoid arthritis, stimulated the accumulation of oxidized Trx upon ROS induction. In-cell NMR readouts of reporter oxidation states revealed that ATG treatment resulted in higher intracellular levels of oxidized Trx in treated versus non-treated cells at similar overall redox conditions i.e. intracellular GSH/GSSG ratios. Thus, the authors confirmed the suitability of this dual reporter approach to assess cellular activities of newly developed Trx reductase inhibitors. The beauty of this system, and the corresponding study, lie in the outlook that it provides for mixed-type in-cell NMR applications. It suggests that active delivery of isotope-labeled targets and natural production of metabolite-based in-cell reporters to simultaneously assess global cellular conditions and individual target response behaviors can be accomplished within the same in-cell NMR sample. By choosing appropriate isotope labeling schemes, such procedures will enable more advanced multiplexed in-cell applications in the future.

5. Metabolite-detected in-cell NMR

NMR detection of cellular metabolites upon drug applications has a long history in the field of *in vivo* NMR (Fig. 1C) [101]. Given the breadth of these studies and the independent nature of the discipline, we will only discuss a few selected examples, which we believe illustrate important new concepts and developments. These primarily relate to efforts to enhance and preserve dynamic nuclear polarization (DNP) effects in different cell types and to develop faster delivery and readout modalities of hyperpolarized metabolites, for which advancements in dissolution DNP (D-DNP) proved essential [102]. We are particularly intrigued by a recent study from the Hilty group in which they combined electroporation, D-DNP and fast shuttling for the delivery of non-permeable, hyperpolarized [^{13}C]-pyruvate into *Saccharomyces cerevisiae* yeast cells [103]. Time-resolved 1D ^{13}C spectra revealed excellent NMR signal qualities of short-lived breakdown products, which enabled Hilty to precisely determine the rates of catabolic pyruvate conversion(s). Given that electroporation is broadly applicable to different cell types and a great variety of substrates, including proteins and DNA (see above), such setups may be employed for multiplexed target-, reporter- and metabolite-observed in-cell NMR approaches in the future. Indeed, two other examples point in this direction. In both cases, metabolic ATP/ADP ^{31}P and glucose/lactate ^{1}H resonances were monitored to evaluate the design of flow-probe perfusion systems for in-cell NMR measurements of protein and DNA targets (see chapter 6) [64,65]. Regarding metabolite-detected in-cell NMR applications, Jacob and Schaefer proved visionary once again. As early as 1985, they studied the metabolism of the ^{13}C- and ^{15}N-isotope labeled broad-spectrum herbicide glyophosate (*N*-phosphomethylglycine) in the soil bacterium *Pseudomonas* by CP-MAS solid-state NMR [104]. Three decades later, Schaefer and Patti revealed the surprising metabolic incorporation of exogenously added ^{13}C-lactate into lipids of cultured mammalian HeLa and H460 cells [105]. They selectively inhibited the reaction with the anti-cancer drug and lactate dehydrogenase (LDH) antagonist oxamate, which enabled them to conclude that this catabolic pathway was strictly lactate dependent and only partially active in ^{13}C-glucose fed cells. Interestingly, Park *et al* observed a similar effect with another LDH inhibitor, galloflavin, when they used in-cell NMR to annotate metabolic changes in HepG2 and NKNT-3 cells upon ^{13}C-glucose labeling [106]. These metabolite approaches interface with cell structure-detected NMR applications that we discuss next.

6. Cell structure-detected NMR

NMR detection of large, isotope-labeled cellular structures such as membranes [107–109], their individual components [51,110],

embedded proteins [49,111–114] and protein complexes [47,115,116], is strongly associated with solid-state (ss) NMR spectroscopy [42]. In-cell ssNMR further revealed details about the intracellular structures of protein aggregates [48] and conformations in bacterial inclusion bodies [40,117]. Even the previously discussed FKBP12 and Trx proteins, which were not detected by solution-state NMR methods in bacteria, yielded interpretable solid-state in-cell NMR spectra [118]. In line with the impressive advancements of ssNMR methods over the past years, especially with regard to developments in proton detection, ultra-fast MAS and DNP methods, the method is now firmly established in in-cell, on-cell and whole-cell NMR applications. As mentioned earlier, detection of NMR-active nuclei in cellular components of whole-cell samples [58,104,119,120] predates multi-dimensional in-cell ssNMR studies by decades [121]. These types of analytical applications continue to provide exciting new insights into the composition of cells and entire unicellular organisms [122,123]. Such detailed *in situ* data are not available from any other method and are powerful for understanding the modes of action of different antibiotics, as well as for the development of new bactericidal and bacteriostatic drugs [11]. Two examples that we want to discuss in this regard are the studies by Separovic and Marcott [124] and Separovic and Sani [125], who used ssNMR detection of the less-frequently exploited ^{2}H deuterium and ^{31}P phosphorus nuclei to investigate the activities of membrane-inserting antimicrobial peptides (AMPs) and antibiotics on the cellular integrities of Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* bacteria. For deuterium-labeling, Separovic and Marcott grew cells in the presence of perdeuterated palmitic acid (d-PA), which is selectively incorporated into saturated membrane phospholipids, without altering the overall membrane compositions of either species. ssNMR detection under low-frequency MAS (10 kHz) allowed them to resolve ^{2}H quadrupole splittings with spinning sideband manifolds directly reporting on lipid acyl-chain dynamics and membrane fluidity. When the AMPs caerin and aurein were added to bacteria, isotropic and spinning sideband intensities changed in a drug and cell-type specific manner. In *Escherichia coli*, both peptides produced a measurable increase in lipid-chain dynamics at their minimal inhibitory concentration (MIC), suggesting that membranes destabilized and permeated in line with known AMP activities. In *Bacillus subtilis,* as expected for a Gram-positive bacterium, quadrupolar splitting was more pronounced, indicative of reduced membrane dynamics and higher levels of order. In contrast to *E. coli*, AMP addition to *Staphylococcus* led to initially decreased lipid-chain mobilities, synonymous with AMP association and membrane rigidification. At AMP concentrations above the MIC, both peptides led to an abrupt increase in membrane dynamics and cell lysis, thus reflecting the fundamental differences of how AMPs interact with Gram-positive versus Gram-negative bacterial cell walls [124]. In a related study, Separovic and Sani employed ^{31}P ssNMR detection to characterize the effects of ampicillin and the AMP maculatin on phosphor-containing cell components such as nucleic acids (DNA and RNA) and phospholipid headgroups of intact *E. coli* bacteria [125]. Both antimicrobial agents induced specific changes in static ^{31}P spectra, which the authors attributed to altered membrane dynamics, increased breakdown and mobility of cellular nucleic acids due to secondary toxicity effects and an overall reduction of metabolic activities. Together, both studies underscore the power of 'alternative' NMR nuclei for obtaining qualitative and quantitative insights into cellular integrity upon drug exposure. Importantly, they also point towards future applications aimed at exploiting multiple NMR-active nuclei to detect different isotope-labeled cell structures in parallel [86,105].

7. In-cell NMR bioreactors

The need to maintain cells under stable metabolic conditions, ideally by continuously supplying them with fresh growth medium, is a core requirement in the field of *in vivo* NMR [8], with different flow-probe bioreactor designs available since the early 1990s [126–128]. In-cell NMR spectroscopists soon realized that these perfusion systems proved equally useful for their own applications targeting intracellular proteins and nucleic acids [62,65,99]. In turn, many of the 'modern' in-cell NMR bioreactor designs were inspired by earlier *in vivo* NMR setups. The majority of current in-cell NMR studies describe *'packing of dense cell slurries into standard NMR sample tubes'*. In these cases, irrespective of whether prokaryotic or eukaryotic in-cell NMR specimens are suspended in pH-buffered nutrient-rich growth media, or simple buffer mixtures, metabolic exhaustion depletes intracellular pools of ATP, glucose and NADPH within ~30 min to 1–2 h [64,65,68,130]. While this does not immediately result in cell death (apoptosis), cells enter a state of metabolic hibernation and reprograming, which affects many of their ATP-dependent activities including those of certain chaperones. Therefore, depletion of cellular nutrients clearly affects the functions of proteins, and probably also their structures (see below). For these reasons, tools to maintain in-cell NMR samples under physiological metabolic conditions are of great importance. One difficulty to overcome with bioreactor systems for in-cell NMR measurements is to ensure that fresh medium can flow freely inside the NMR sample tube without washing out the cellular specimens and without them obstructing inlet and outlet channels. To this end, cells are often embedded in porous support materials through which growth medium can pass but which are too large to clog the respective perfusion paths (Fig. 2A). In 2008, the group of Pielak introduced the first such device for protein in-cell NMR measurements of bacterial cells suspended in alginate microcapsules, the 'circulating encapsulated cell' (CEC) bioreactor (Fig. 2B) [26,69]. In 2017, Ito and Kigawa successfully adapted the alginate encapsulation scheme for mammalian cells [67]. Different to Pielak's approach, they produced embedded cells by capillary centrifugation [129] and performed in-cell NMR measurements under continuous flow conditions, without moving cells between mixing and detection chambers as in the CEC setup (Fig. 2C). By studying the human adenylate kinase 1 (hADK1), they found that intracellular hADK1 rapidly unfolded when perfusion was switched off, whereas the protein remained folded when fresh medium was supplied [67]. These results provide a strong argument for the importance of the cellular energy metabolism in preserving the structural integrity of certain classes of folded proteins. In 2013, Ogino and Shimada introduced the use of a synthetic, thermo-reversible hydrogel (Mebiol) to embed mammalian cells for in-cell NMR measurements (Fig. 2D) [68]. In turn, they performed [^{1}H–^{13}C]-methyl TROSY experiments on the 9 kDa, selectively ^{13}C isoleucine-, leucine- and valine- (ILV) labeled, cytoskeleton-associated glycin-rich domain (CAP-Gly1) of the cytoplasmic linker protein 150 (CLIP-150), to demonstrate that their system ensured greatly diminished protein leakage from manipulated cells and yielded superior quality in-cell NMR spectra [68]. The same bioreactor was later used in the dual Trx-target, GSH/GSSG metabolite-reporter study by Nichida and Shimada that we discussed earlier [99]. The groups of Shekhtman and Fonseca & Cruz devised similar encapsulation schemes based on low-melting agarose (Fig. 2E) [64,130]. Shekhtman investigated RNA-mediated quinary structure effects on Trx in agarose-embedded bacterial (*E. coli*) and mammalian (HeLa) cells [130]. Specifically, they asked whether perfusing cells with antibiotics that target ribosomes and compete with mRNA binding to ribosomes, resulted in measurable

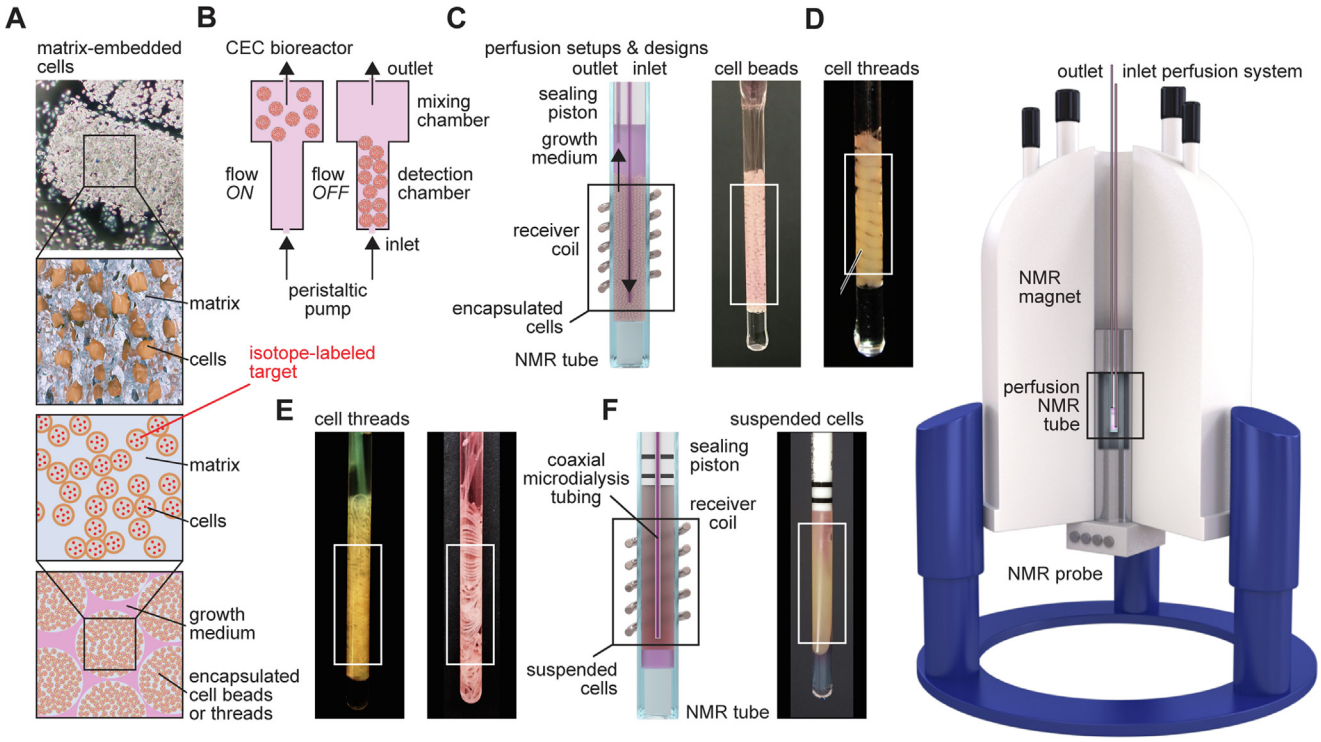

Fig. 2. Outline of current bioreactor designs for in-cell NMR studies. (A) Illustration of gel-encapsulated cells harboring isotope-labeled NMR targets. Perfusion of cells embedded in different porous matrix materials with growth media enables the continuous exchange of nutrients to preserve cellular viability and physiological energy metabolism. The microscopy image on the top shows encapsulated cells in a low-melting agarose gel-thread (reproduced from [130]). Cell matrices may be molded into different shapes and forms. (B) Overview of the circulating encapsulated cell (CEC) bioreactor by Pielak et al. [69]. Under flow conditions, embedded cells are pushed into an upper mixing chamber for optimal medium exchange. When the flow is switched off, cells settle into the lower NMR detection volume for data acquisition. (C) Schematic outline of an NMR sample tube for measurements in a flow-probe perfusion setup. Capillary inlets and outlets enable the flow of growth medium. Embedding of cells in large macromolecular structures such as beads or threads prevents clogging of the system. With these devices, in-cell NMR spectra can be recorded in a continuous manner. The image on the right shows the bioreactor setup by Ito and Kigawa, reproduced from [67]. (D) Image of the bioreactor setup by Shimada et al. adapted from [68]. (E) Alternative bioreactor designs and embedded cell threads by Shekhtman (left) and Cruz (right), reproduced from [130] and [64], respectively. (F) Micro-dialysis bioreactor setup by Luchinat and Ravera, as reported in [65]. In contrast to designs shown in panels C-E, cells are directly dispensed to the volume outside the central coaxial dialysis chamber. The schematic drawing on the right depicts the position of the respective bioreactor sample tubes inside the NMR spectrometer. Technical illustrations of bioreactors and the NMR spectrometer by Barth van Rossum.

modulations of quinary structure interactions between cellular RNA and Trx [22,131]. They found that addition of streptomycin and tetracyline (targeting the 30S ribosomal subunit) enhanced Trx-RNA binding and led to severe line-broadening of protein in-cell NMR spectra. By contrast, chloramphenicol exposure (which targets the 50S subunit) had no effect [130]. Based on these findings they concluded that weakening of ribosome-mRNA interactions with antibiotics exacerbated quinary structure interactions of intracellular proteins with RNA. Fonseca and Cruz employed ^{31}P NMR readouts to investigate cytotoxicity effects of the anti-cancer drugs 5-fluorouracil (5-FU) and the calixarene derivative Calix-NH_2 on agarose-embedded HeLa cells [64]. An altogether different bioreactor setup was introduced by Luchinat and Rivera [65]. Rather than embedding cells in a solid-support matrix, their system makes use of a central micro-dialysis chamber through which fresh medium flows and nutrients are exchanged (Fig. 2F). Non-encapsulated cells harboring isotope-labeled proteins are added to the volume outside the dialysis membrane for in-cell NMR measurements. Using this setup, they recorded ^{1}H and ^{31}P NMR spectra on human HEK293T cells to confirm stable glucose, lactate and cellular ATP levels for up to 2 days [65]. In turn, they showed that their bioreactor preserved the physiological, single-metal coordination state of over-expressed, ^{15}N isotope-labeled human super oxide dismutase 1 (SOD1) for more than 12 h, whereas non-native, double Zn-bound SOD1 accumulated when perfusion was switched off.

Of all the in-cell NMR bioreactor designs discussed here, the dialysis concept appears most promising, especially because cells can be added and removed easily without prior embedding procedures. This may prove particularly useful when additional assays, by cellular microscopy for example, are to be performed before or after, or even in parallel to in-cell NMR measurements, as recently shown by Eliceiri and Fain for DNP-enhanced NMR and imaging studies of metabolites in 3D cell cultures [66]. Indeed, reusable or disposable bioreactor hardware components that are compatible with commercial flow-probe setups would allow such perfusion systems to be installed on a wide range of NMR spectrometers [132]. Industry efforts in this direction are underway and we are excited about the possibilities that these systems will offer in the future.

8. Conclusions and perspectives

We hope to have conveyed a comprehensive overview of cellular NMR applications and their prospects for drug discovery applications. As we have outlined in this review, the breadth of cellular systems amenable to such analyses ranging from prokaryotic to eukaryotic cells is remarkable. Paired with the recent advances in bioreactor technologies, they allow researchers to maintain cells under tightly controlled metabolic conditions, irrespective of the cells' origins and their specific growth requirements in terms of

media and additives. While many of the tools and applications that we discussed have been developed for unique cellular systems, we believe that most of the ideas and concepts reviewed here are easily transferrable to other types of cells and approaches. For these reasons, we also list studies in Table 1 that were not directly geared towards drug investigations, because we feel that they offer exciting possibilities in these directions [65,67,90,103,133–135]. As can be appreciated in Table 1, drug-related cellular NMR studies are thriving and a trend towards multiplexed approaches is evident. By choosing appropriate isotope labeling and corresponding NMR-detection schemes, we will be able to observe multiple NMR-active nuclei in different cellular and non-cellular structures simultaneously and obtain target, compound, metabolite, or reporter-specific readouts in parallel. With such multiplexed approaches, we will be able to correlate metabolic cell states (cytotoxicity) with compound integrity, drug-target interactions (specificity) and respective modulatory activities (efficacy). What is also evident in Table 1 is the underutilization of the isotope-repertoire in drug-detected in-cell NMR studies, with few exceptions [73,86]. Clearly, there is ample room for future investigations with other NMR-active nuclei, of which ^{19}F fluorine probably offers the most promising option. As we mentioned earlier, we are particularly intrigued by recent breakthroughs in bioreactor designs and believe they provide unparalleled advantages for cellular NMR approaches, especially in terms of robustness and reproducibility. By benchmarking bioreactor systems on different NMR spectrometers and for different cells under different conditions, we will be able to define industry standards and guidelines for their best possible use in the future.

Acknowledgments

We thank Carla Cruz, Enrico Luchinat, Takanori Kigawa, Alexander Shekhtman and Ichio Shimada for sharing original images of their in-cell NMR bioreactor setups, and Barth van Rossum for technical illustrations. P.S. acknowledges funding by the ERC Consolidator Grant #647474 (NeuroInCellNMR).

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