Published in final edited form as:

Prog Nucl Magn Reson Spectrosc. 2013 April ; 70: 25–49. doi:10.1016/j.pnmrs.2012.10.001.

New Frontiers and Developing Applications in ^{19}F NMR

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Keywords

fluorine; NMR; pH; β -galactosidase; metal ions; hypoxia; enzyme activity

1 Introduction

1.1 Overview

^{19}F NMR is gaining interest as a tool for diverse physiological and pharmaceutical investigations as evidenced by new reporter molecules and detection strategies. The relatively high sensitivity of ^{19}F and lack of interfering background signal in the body have enabled the observation of exogenously administered agents and their metabolites. The ^{19}F nucleus exhibits a large chemical shift range (~300 ppm), which is exquisitely sensitive to the electronic microenvironment, and many reporter molecules have been developed. In addition to chemical shift studies, relaxation processes (R_1 and R_2), and chemical exchange have also been tailored to be responsive to a parameter of interest such as pO_2 , pH, metal ion concentrations, transgene/enzyme activity or hypoxia. Recent emphasis has been on enhancing signal sensitivity and developing novel response mechanisms to sense environmental parameters. This review focuses on quantitative tissue oximetry, detection of ions including pH, and detection of enzyme activity. Fluorine NMR has been widely exploited for laboratory investigations and is garnering developing use in pre-clinical applications for small animals *in vivo*.

1.2 Background

Fluorine NMR offers many attractive features, most notably detection sensitivity approaching that of protons. ^{19}F has a nuclear spin $I=1/2$, a gyromagnetic ratio of 40.05 MHz/T, and is 100% naturally abundant [1]. The high gyromagnetic ratio often allows the use of existing proton NMR instrumentation with the minimum of component adjustments. ^{19}F NMR is particularly attractive for *in vivo* applications since there is essentially no endogenous signal from tissues. Fluorine does occur extensively in bones and teeth, but the solid matrix causes rapid transverse relaxation (high R_2^*) and very broad signals, which can either be removed by deconvolution or by use of appropriate pulse sequences. Thus, fluorinated reporter molecules or drugs may be introduced into the body and detected readily with high sensitivity and without background interference.

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Fluorine NMR typically requires millimolar concentrations of reporter molecules, as opposed to picomolar typical of the PET tracer ^{18}F [2]. However, the lack of radioactivity for ^{19}F makes its use much simpler; notably, molecules may be synthesized and stored avoiding the need for the rapid synthesis and immediate application associated with the 110 min half-life of ^{18}F . Agents labeled with ^{19}F may be traced over hours to days and even weeks allowing assessment of long term pharmacokinetics or evolution of pathophysiology, such as hypoxiation accompanying tumor growth [3–6]. Recently, ^{19}F NMR has found a niche application in tracking stem cells *in vivo* [7–14]. Labeled cells provide positive signal, as opposed to the signal loss historically associated with iron-oxide labeled cells [15], potentially improving detectability in heterogeneous environments.

^{19}F is exceptionally sensitive to molecular and microenvironmental changes as exemplified by the many ^{19}F -based reporter molecules designed to interrogate physiological phenomena *in vivo* (e.g., Table 1, Fig. 1). ^{19}F NMR reporter agents have exploited diverse parameters including signal intensity (SI), chemical shift (δ), and relaxation rates ($R_1 = 1/T_1$) and ($R_2^* = 1/T_2^*$). Indeed, each of these parameters has been exploited for specific ^{19}F NMR reporter molecules (Table 1). It should be noted that the International Union of Pure and Applied Chemistry (IUPAC) ^{19}F NMR chemical shift standard is fluorotrichloromethane (CFCl_3) [16]. However, this volatile solvent is not convenient for biomedical applications and many biological investigations have used sodium trifluoroacetate ($\text{CF}_3\text{CO}_2\text{Na}$ or NaTFA ; $\Delta\delta^{\text{TM}}$ versus CFCl_3 -76.530 ppm), as we do here. Sodium trifluoroacetate has the advantage of being readily available, inexpensive (about \$1/g), quite non-toxic ($\text{LD}_{50} > 2$ g/kg in mice) and may be used as either an external, or internal chemical shift standard in biological investigations [17–19]. Fluorine chemical shifts can be quite unpredictable, but compilations of ^{19}F NMR chemical shifts and theoretical predictions have been reported [1, 20–22]. Fluorine coupling constants may also appear unusual, since they often do not decrease monotonically with number of bonds, complicating molecular analysis [23–26].

The presence of the ^{19}F atom may modulate molecular properties, most notably hydrophobicity and this becomes more significant for multiple fluorines, as encountered in CF_3 groups. In addition, the electronegativity can alter ionization of adjacent groups such as carboxyl, phenol, and amine (this is explored further in the next section and presented in Table 2). Several pharmaceuticals, agrochemicals and pesticides include a fluorine atom and have been examined by ^{19}F NMR as reviewed previously [27, 28]. In other cases ^{19}F may be added to explore structure activity relationships or the ADMET (Absorption, Distribution, Metabolism, and Excretion Toxicity) process [29]. A recent review [30] explores many different applications of ^{19}F NMR to chemical biology including xenobiotic metabolism, protein structural folding, and enzyme mechanisms. Fluorinated amino acids (e.g., F-tryptophan, F-tyrosine, difluoromethionine, trifluoroleucine, pentafluoroleucine, and F-serine) have been used to interrogate molecular structure, bindings conformations, microenvironment and potentially modify specific binding sites [31–36]. A recent innovation exploited SF_6 as a “spy molecule” based on chemical shift and intermolecular ^1H heteronuclear Overhauser effects when accessing molecular pockets [37].

Historically, most drug studies have examined pharmacokinetics of fluoropyrimidines, particularly, 5-fluorouracil (5-FU) to reveal dynamics of uptake, biodistribution, and metabolism *in vivo*, as reviewed by others [29, 38–41]. 5-FU has a narrow window of efficacy and there has been much interest in optimizing efficacy while reducing toxicity. Wolf *et al.* found that “trappers” tended to show better response [41], though a recent report is potentially contradictory [42]. Competing reactions convert 5-FU to the active cytotoxic 5-fluoronucleotides (FNuct), or to less cytotoxic catabolites such as 5,6-dihydrofluorouracil (5-DHFU) and α -fluoro β -alanine (FBAL) [39, 41, 43]. Relative tumor and liver retentions appear to influence efficacy and toxicity and have been extensively investigated in both

animal models and patients [41, 42, 44–46]. The retention of 5-FU is reported to be considerably enhanced in tumors with lower pH [44–46] prompting investigations of the ability to alter pharmacokinetics by modulation of tumor pH [47, 48]. Since the chemical shifts of some fluoronucleotides derived from 5-FU are sensitive to pH they could potentially be used to measure intracellular pH (pHi) directly *in vivo*, although the presence of a mixture of products may complicate interpretation [46, 49, 50]. Generally, ^{31}P NMR has been used to assess the tumor pH [44]

One approach to mitigating toxicity and enhancing tumor selectivity is the use of pro-drugs in conjunction with gene therapy. Specifically, cytosine deaminase converts the relatively innocuous 5-fluorocytosine (5-FC) to 5-FU. This has been observed as a ~2 ppm chemical shift using ^{19}F NMR in mice or rats and is discussed further in Section 4 [51–54].

Various other drugs include fluorine atoms [55] and NMR studies have been reported for gemcitabine [56, 57] and flutamide [58]. Beyond examining cancer chemotherapeutics ^{19}F NMR has been applied to various psychiatric agents in animals or humans, notably, fluoxetine, fluvoxamine dextfenfluramine, haloperidol decanoate, and paroxetine [59–67]. Several gaseous anesthetics are fluorinated (*e.g.*, halothane, enflurane isoflurane, sevoflurane, and desflurane) allowing NMR spectroscopy in mice or man, but imaging is difficult due to the very short T_2^* values associated with membrane binding [68–73].

While ^{19}F has many virtues it is important to place it in the context of other nuclei. Proton MR is ideal for anatomical imaging because of the enormous water signal and multiple contrast mechanisms indicating tissue boundaries and pathology [74]. It has become the mainstay of clinical radiology and becomes even more attractive with recent concerns over potential radiation toxicity of computed tomography (CT) [75]. Furthermore, so-called functional MRI [76] offers insight into physiology and pathophysiology based on contrast derived from flow, diffusion and endogenous paramagnetic species, such as iron [77, 78], oxygen [79, 80] and deoxyhemoglobin [81–83]. Exploiting endogenous signals is attractive, but apart from detection of the proton water resonance, other materials or reporter molecules are masked by the intense and essentially ubiquitous water. Many techniques have been developed for effective water suppression ranging from selective multiple quantum spectroscopy to brute force saturation or selective evolution of spins following multi-pulse excitation [84, 85]. Indeed ^1H spectroscopy does find extensive application in diagnosing brain tumors [86, 87] and continues to be explored in other disease sites such as prostate [88] and breast [89]. Water may be replaced using D_2O , though this is impractical in most biological systems and may cause growth delay [90].

Alternatively, heteronuclei may be assessed based on natural presence or specific administration. Sodium ions (^{23}Na) are prevalent and detectable, but generally present a single resonance and chemical shift agents are required to simply differentiate intra- and extracellular signals [91, 92]. ^{31}P has provided insight into several so-called high energy metabolites vital for metabolism and homeostasis, notably adenosine triphosphate (ATP), phosphocreatine (PCr), and inorganic phosphate (Pi), which is additionally responsive to pH [93–95]. While ^{31}P was enthusiastically applied in animal and human studies for some years, the poor spatial resolution dictated by low sensitivity has diminished use. Isotopic substitution can increase signal intensity with minimal perturbation of normal metabolic pathways. Deuterium enrichment has been applied potentially allowing 6,400 fold signal gain, though it is subject to kinetic isotope effects [96, 97]. Carbon-13 enrichment is widely applied with minimal kinetic isotope effects, potential 100-fold signal gain and wide chemical shift range so that multiple molecules may be detected simultaneously. Together, deuterium and ^{13}C have been widely applied to the analysis of metabolic pathways based on isotopomer analysis, which can reveal both the source and fate of molecular moieties [98,

99]. Enrichment provides ready discrimination of labeled molecules versus endogenous background, but even with enrichment ^{13}C is not particularly sensitive precluding widespread use hitherto except in perfused organs. New high field magnets (*e.g.*, 7 T) for humans promise new opportunities in terms of acquisition times and spatial resolution [100]. Recently, hyperpolarization has been reported to yield as much as 60,000 fold signal enhancement [101, 102] providing new opportunities. However, the signal is transient, decaying with T_1 , which introduces similar hurdles to those encountered with use of radioactive nuclei such as ^{11}C in non-NMR studies: rapid decay precludes sample storage and limits studies to seconds or minutes after infusion. Of course hyperpolarized ^{13}C has the distinct advantage of providing spectral resolution, which is unavailable with radionuclide imaging approaches.

Applications of ^{19}F NMR and developments in the field have been reviewed frequently and the reader is directed to earlier reviews for background [28, 35, 103]. Notably, some reviews have focused on specific topics such as applications to pharmacology [29, 104], physiology [105], reporter molecules [27, 106–110]. To broaden the utility of ^{19}F NMR various chemical, physical, and pharmacological innovations have been applied to enhance signal to noise, as discussed in the next section.

2 Innovations and Enhancements

2.1 Chemistry

Chemical approaches to enhance ^{19}F signal can take various forms. The simplest may be adding additional equivalent fluorine atoms [111, 112]. A more subtle approach recently exploited paramagnetic relaxation enhancement (PRE) based on spatial proximity to a relaxing paramagnetic lanthanide center [113]: furthermore, enzyme induced molecular cleavage or pH dependant conformational changes may alter the proximity and signal characteristics [109]. Alternatively transverse relaxation may be enhanced by formation of densely packed molecular agglomerations, potentially liquid crystal type structures with restricted motion causing severe ^{19}F line broadening [112, 114]. Structures have been designed whereby interaction with a target may cause disaggregation of the supramolecular structure resulting in signal “turn-on” due to a volume phase transition: notably, protonation or binding of specific target protein to a tethered ligand causes disassembly and signal modulation [112, 115].

NMR can provide quantitative measurements based on signal integration. In principle, the larger the number of equivalent ^{19}F atoms the stronger the signal (Figs. 1 and 2, Table 2). Two fluorines may be introduced as a geminal alkyl pair as demonstrated by Deutsch *et al.* in a series of pH reporters [116]. However, for 3,3-difluoro-2-methyl alanine the two fluorines are magnetically non-equivalent and exhibit an AB quartet and thus, the signal per resonance line is actually lower than for a single fluorine atom, particularly when proton decoupled (Table 2). Two equivalent fluorines have been presented in structures such as 5F-BAPTA (Fig. 1) based on symmetry of aromatic rings [117]. Trifluoromethyl moieties are popular since they are metabolically inactive, provide a single non-coupled signal and have steric volume similar to an isopropyl group [1]. Four equivalent fluorines are found in symmetrically para substituted phenols, benzoic acids, and anilines, although differential substitution removes the symmetry yielding pairs of resonances [118]. The recently used pentafluorosulphonyl moiety ($-\text{SF}_5$) has an asymmetric structure with four equivalent fluorines displaying a doublet ($J_{\text{F,F}} \sim 150$ Hz) from coupling to the fifth fluorine [1]. Six equivalent fluorines are observed for an isopropyl group as in the nitroimidazole hypoxia reporter CCI-103F [119]. Hexafluorobenzene also exhibits a single resonance, which has been exploited *in vivo* for oximetry (Table 1, Fig. 1). A tris trifluoromethyl (t-butyl) group was recently developed and used to investigate nucleic acid conformation [120] and so-

called (nonafluoro-*tert*-butoxy)methyl ponytails are generating increased attention [121]. Twelve equivalent fluorines were used by Takaoka *et al.* [112], who evaluated the relative merits of varying the number of fluorine atoms (Fig. 2). Perfluorocrown ethers show a single resonance and various ring sizes have been reported, with 15C5 (n=20 F) being used as a cell labeling agent and for *in vivo* oximetry [7, 122]. Another agent with 27 equivalent fluorines was reported and used for *in vivo* studies in mice [123].

In terms of NMR detection, the more equivalent fluorines the stronger the signal. However, fluorine modulates molecular properties, since the fluorine atom is strongly electronegative and the CF bond strongly polarized [124]. Various examples have been reported for molecular series with increasing fluorination, *e.g.*, modulation of pKa for the series of acetic acids $\text{pKa}(\text{CH}_3\text{CO}_2\text{H}) = 4.76$, $\text{pKa}(\text{CH}_2\text{FCO}_2\text{H}) = 2.59$, $\text{pKa}(\text{CHF}_2\text{CO}_2\text{H}) = 1.24$ and $\text{pKa}(\text{CF}_3\text{CO}_2\text{H}) = 0.23$ [125]; the series of primary amines $\text{pKa}(\text{CH}_3\text{CH}_2\text{NH}_2) = 10.7$, $\text{pKa}(\text{CH}_2\text{FCH}_2\text{NH}_2) = 9.0$, $\text{pKa}(\text{CHF}_2\text{CH}_2\text{NH}_2) = 7.3$ and $\text{pKa}(\text{CF}_3\text{CH}_2\text{NH}_2) = 5.7$ [126] and the series of fluoroalanines developed as pH reporter agents (Table 2, [127]). pKa values have been calculated for meta fluoro substituted benzoic acids such that $\text{pKa } m\text{-SF}_5\text{ArCO}_2\text{H} = 4.82 < m\text{-CF}_3\text{ArCO}_2\text{H} < m\text{-SCF}_3\text{ArCO}_2\text{H} < m\text{-OCF}_3\text{ArCO}_2\text{H} < m\text{-FArCO}_2\text{H} = 5.28$ [128, 129]. The extent of fluorination also alters the hydrophobicity and ability of molecules to cross membranes, such as the blood brain barrier, a crucial consideration for anesthetics and psychiatric drugs [130, 131]. From the NMR perspective the fluorine chemical shift in a CF_3 moiety is less sensitive to its chemical environment than a directly bonded fluorine atom since the electronic perturbation must pass through an additional carbon-fluorine bond (compare 6-FPOL vs. CF_3POL , PFONP vs. $p\text{CF}_3\text{ONP}$ and PFONPG vs. $p\text{CF}_3\text{ONPG}$ in Table 2) [111]. We have measured the acid-base chemical shift of $p\text{-SF}_5\text{ArOH}$ (4-hydroxyphenylsulfurpentafluoride) to be only 1.8 ppm for the F_4 doublet, though 3.9 ppm for the downfield quintet from this AX_4 spin-system. By analogy with ^{18}F PET, hypoxia has been detected using ^{19}F -nitroimidazoles [132–134]. In an effort to enhance SNR (signal to noise ratio) additional F atoms have been incorporated (Table 2) with minimal perturbation to the active nitroimidazole [119, 135–140]. However, additional F atoms reduce the water solubility (incising octanol/water partition coefficients) making *in vivo* use more difficult [132].

Increased numbers of equivalent fluorine atoms can also be achieved using derivatized polymers with multiple labels or micelle formulations [141–146]. Perfluorocarbons (PFCs) have been incorporated into various nanostructures and appropriate shells allow targeting, cell tracking and enhanced relaxation [7, 8, 10, 147–151]. Simple incubation of cells with PFC emulsion can label them and allow tracking *in vivo* [7]. The concept is being developed by Celsense of Pittsburgh (www.celsense.com) and has been demonstrated in various cells, tissues and locations [7–14]. Since various PFCs can form stable emulsions different cell populations can be uniquely labeled and observed with spectral selective imaging allowing assay of distributions, as shown by the Wickline group (Fig. 3) [10].

Formation of membranes, micelle structures, or particles can restrict molecular mobility influencing NMR visibility: as noted, solid state materials tend to have very broad signals [152]. Tight packing of PFC into polymer micelles can reduce their ^{19}F NMR visibility, but modification such as swelling induced by addition of DMSO increases mobility and observability, as demonstrated for perfluorocarbon-loaded shell cross-linked knedel-like nanoparticles [114]. Likewise, it was recently reported that dense fluorinated nanogels are ^{19}F NMR “silent”, but a hydrophilic–hydrophobic (volume-phase) transition of the polyamine gel core in response to protonation could “turn-on” the signal. Gels have been described, which are pH sensitive exhibiting a change in T_2 from < 1 ms at pH 7.4 to > 50 ms at pH 6.5 [115, 153]. Disassembly of nanoprobes and ^{19}F NMR detectability may also be caused by association with specific proteins revealing molecular recognition, as discussed

further in Section 4 [154]. An example is shown in Fig. 2, which both reveals molecular recognition and the enhanced signal available from additional fluorine atoms. No ^{19}F signals are detected from immobilized PFC in the nanoparticles making it easy to identify signal from the protein-ligand complex.

Altering spin lattice relaxation can enhance the efficiency of data acquisition. In some cases addition of Gd-DTPA to an aqueous solution reduces T_1 and hence allows faster acquisition [155–158]. Indeed, this has been used to identify cellular compartmentation based on the ability of the contrast agent to relax extracellular, but not intracellular material [159]. Direct relaxation can also be achieved by including F or CF_3 in a paramagnetic ligand complex [113, 160]. If ^{19}F is attached very close to a paramagnetic center, such as Gd^{3+} or Fe^{3+} , the local paramagnetic relaxation effect (PRE) can strongly influence the fluorine atom. Close proximity essentially quenches ^{19}F NMR signal, but activatable separation makes the signal visible. This is quite analogous to optical FRET (Förster (fluorescence) resonance energy transfer), where proximity controls signal. Molecular structures are crucial to generate optimal proximity relevant to a particular lanthanide [113, 161–163]. The proximity may be altered by molecular cleavage revealing enzyme activity as discussed in Section 4 [164–166]. Molecular orientation may also be affected by protonation and pH-enhanced relaxation has been reported [167, 168].

Increasingly, ^{19}F is incorporated into nanoparticles or molecular aggregations. Several studies have demonstrated that incorporation of a lanthanide ion such as Gd^{3+} into nanoparticle shell enhances R_1 and increases the SNR [149, 169]. The presence of oxygen enhances relaxation and this is widely exploited for *in vivo* oximetry, as described in Section 3.1. Significantly, PFC relaxation is essentially invariant with Gd^{3+} ions in surrounding solution [170] and clearly proximity and the surface to volume ratio are crucial since dipole-dipole relaxation falls with $1/r^6$.

2.2 Physics

In addition to modifying the chemical structure to enhance signal to noise, and thus detection, advances in MR physics have played a role. For *in vitro* studies highly concentrated samples may be used, but solubility and toxicity generally preclude increasing doses *in vivo*. Generally, the use of higher magnetic fields provides greater signal to noise, though the theoretical gain may not be realized because of quadrupolar broadening or increased T_1 and shortened T_2 .

Data acquisition efficiency can be enhanced by rapid small flip-angle excitation imaging using gradient echoes (*e.g.*, FLASH) or ultimately echo planar or echo volume methods, though the one pulse imaging methods are often subject to greater spatial distortions [122, 171, 172]. As discussed in Section 3.1, we use EPI (Echo Planar Imaging) for ^{19}F MRI oximetry (FREEDOM- Fluorocarbon Relaxometry Using Echo Planar Imaging for Dynamic Oxygen Mapping) [173], providing pO_2 maps with 6.5 minute temporal resolution and recently an accelerated approach was demonstrated using a Look-Locker acquisition [174]. Of course fewer acquisitions or fewer relaxation points can also enhance temporal resolution, ultimately provided by simple two-point partial saturation [27, 175]. Inevitably, fewer data points provide poor curve-fit reliability and may compromise the accuracy of relaxation time measurements [176]. A new approach for accelerated imaging is compressed sensing, whereby sparse signals may be adequately detected despite under sampling [177]. Despite apparent conflict with the Nyquist requirements, robust investigation in terms of spatial distribution and spectral resolution is widely reported for proton MRI and recent examples show accelerated ^{19}F MRI [178, 179].

Interleaved detection of several spectral lines was recently presented for detecting 5-FU and its metabolites in mice at 9.4 T with validated metabolite quantification [180]. Ultimately, ^{19}F and ^1H MRI may be acquired simultaneously with dual tuned coils, particularly at low field [181], though the gain in efficacy may be relatively modest, since ^1H MRI is already rapid and it is the ^{19}F alone which is often rate limiting. We often find that the added versatility of double tuned or even tunable coils provides suboptimal detection at the less sensitive frequency. However, dual acquisition should be useful to allow simultaneous detection of dynamic changes, *e.g.*, pharmacokinetics of a fluorinated drug (uptake, clearance, and metabolism) together with pharmacodynamics of its effect (*e.g.*, vascular perturbation). In principle, this is the goal for PET/MRI [182, 183].

Combined modality reporter agents are gaining popularity though often for parallel, rather than simultaneous use. Recent reports have shown evolution of both color pigment or fluorescence and ^{19}F NMR signal [109, 184, 185]. New β -gal (β -galactosidase) reporters simultaneously show ^{19}F chemical shift and ^1H contrast effects [186, 187].

2.3 Pharmacology: targeting

Small molecules may distribute indiscriminately throughout the body requiring overall millimolar concentrations. Signal enhancement may be achieved by targeting agents to a tissue/volume of interest and retention. The simplest approach is direct injection, as used for hexafluorobenzene (HFB) in tissue oximetry [173, 188], but systemic administration with local accumulation is less invasive. Perfluorocarbon emulsions may exhibit prolonged vascular retention, but ultimately about 90% clears through the reticuloendothelial system (RES) [189]. While this is problematical for labeling organs such as the heart or tumors [4, 175], which may require multiple doses, it makes labeling the liver and spleen very efficient [190]. Active targeting has been demonstrated by the Wickline group showing various nanobeacons with activated surfaces targeting specific receptors such as $\alpha_v\beta_3$ -integrin generating accumulation at sites of inflammation such as atherosclerotic plaques [169] and inflamed kidneys [110, 150]. A potential problem is the long-term retention of unbound PFC in the vasculature creating a large non-specific background signal and potentially masking the binding selectivity. An ingenious solution has been demonstrated exploiting differential diffusion, noting that bound PFC nanoparticles are immobilized [191]. Flowing versus stationary spins has also been exploited in the so-called BESR method, whereby new relaxed spins enter the region of interest minimizing saturation effects [192].

Cells may be labeled *in vitro* prior to placement into animals allowing their location and migration to be tracked following implantation *in vivo* [7, 9, 10, 13]. In small animals such tracking may be more effectively achieved by transfection with reporter genes such as luciferase or fluorescent proteins, which have the advantage of propagating during cell divisions [193]. Where optical imaging is unsatisfactory, or transfection is inappropriate, the direct cell labeling can be effective and MRI is more appropriate for larger animals and potentially man.

3 Pathophysiology

3.1 Oximetry

Quantitative ^{19}F MR oximetry has been developed over many years based on the sensitivity of the spin lattice relaxation rates of perfluorocarbons (PFCs) to oxygen [194]. PFCs have remarkable properties with very high gas solubility and high density, while being hydrophobic, essentially inert, and non-toxic. For oximetry the most crucial property is the ideal liquid gas interaction with oxygen giving a linear dependence for the ^{19}F spin lattice relaxation rate $R_1 = A + B \text{ pO}_2$, as reported and summarized for many different PFCs [105, 173]. The relationship remains linear across the whole range of pO_2 values including

hyperbaric conditions [195]. Since PFCs are exceedingly hydrophobic, ions and proteins do not dissolve and therefore calibration curves established *in vitro* may be used *in vivo* [170, 196–198]. However, the free radical TEMPO laurate was found to influence relaxation rates [196] and Gd ions attached to the surface of PFC nanoparticles influenced R_1 [149]. The sensitivity of R_1 to pO_2 is both field and temperature dependent, and thus, pertinent calibration curves are required (Table 3) [25, 188, 198]. The temperature dependence means that even a relatively small error in temperature estimate can introduce a sizable discrepancy into the apparent pO_2 . Relative potential errors are indicated in Table 3; specifically a 1 °C error in temperature estimate would lead to an 8 torr/°C error for perfluorotributylamine [198], 3 torr/°C for PFOB (perflubron) [199] or 15-crown-5-ether [122] and 0.1 torr/°C for hexafluorobenzene (HFB) [188] when pO_2 is actually 5 torr.

For *in vivo* oximetry the reporter must be delivered to the region of interest. This may be achieved by direct injection into the tissue of interest or systemic delivery. Liquid PFCs may be injected directly into tissues, but they must be emulsified prior to intravenous administration. Stable emulsions depend critically on formulation and vapor pressure of the PFC, but potential commercial synthetic blood substitutes [200, 201] and ultrasound contrast agents [202, 203] have been developed. Recently, several laboratories have generated emulsions and nanoparticles including targeted agents [13, 110, 147, 148, 151, 191]. Following intra venous (IV) infusion, a typical blood substitute emulsion circulates with a half-life of 12 h providing substantial clearance within two days [204]. Several reports examined tissue vascular pO_2 , while PFC remained in the blood [197, 205–208]. The particulate nature of PFCs leads to extensive macrophage uptake and sequestration in the reticuloendothelial system. Therefore oximetry is particularly efficient in the liver and spleen with reports examining pO_2 response to oxygen breathing challenge or the influence of von Hippel-Lindau (VHL) expression and inactivation in transgenic mice [122, 176, 190, 209]. Tissue retention allows non-invasive oximetry *in vivo* over a period of weeks. ^{19}F oximetry has been reported in mice, rats, rabbits, cat, pigs and man as indicated in Table 1. Multiple repeat systemic doses were required to generate sufficient SNR in tissues with lower accumulation [189], such as the heart [175, 210] or tumors [4, 199, 211] resulting in extensive hepatomegaly and splenomegaly [212]. Both spectroscopy and imaging have been applied to perfused rat hearts labeled with sequestered PFC emulsion [175, 210] and a direct correlation was observed between loss of mechanical function (developed pressure) and hypoxiation following total global ischemia (TGI) [27]. ^{19}F NMR oximetry has been used to investigate tumors with respect to both acute studies of interventions and chronic studies of growth following IV administration of PFC emulsions and vascular clearance [4, 5, 122, 211, 213–219]. However, we believe a crucial caveat is that PFC emulsion uptake and deposition in tumors is highly variable and heterogeneous with most signal occurring in well perfused regions [4, 218]. A recent study combined diffusion weighted proton MRI with ^{19}F oximetry, using the ADC to characterize the tissues where oximetry occurred [220]. Following sequestration, bulk PFC does not seem to redistribute within tissue, but remains associated with specific locations allowing chronic studies during tumor development and revealing progressive hypoxiation [4, 5].

Direct injection of PFC into tissue allows immediate interrogation of regions of interest without the need for vascular clearance or bias towards well vascularized regions. The use of a fine sharp needle and small volumes of the reporter molecule is minimally invasive and these procedures have been reported in multiple tissues allowing measurement of oxygenation in the retina [221–223], cerebral interstitial and ventricular spaces [224], brain, kidney, liver, gut, and muscle [225–227]. We have undertaken extensive studies using direct intra-tumoral (IT) injection of neat hexafluorobenzene (HFB) and other research teams have also recently reported such studies [173, 174, 218, 228–231]. Volumes as small as 10 μ l HFB provide adequate signal for spectroscopy and precise pO_2 measurements [188];

although the NMR measurement may be non-localized the discrete reporter location provides a highly localized measurement. However, tumors are recognized to exhibit heterogeneity [134, 232] and thus imaging appears crucial to map pO_2 distributions successfully (Fig. 4). To provide effective temporal resolution for dynamic measurements we developed *FREDOM* [173], which typically provides 50 to 150 individual pO_2 measurements (voxels) across a tumor simultaneously in about 6.5 minutes with a precision of 1 to 3 torr in relatively hypoxic regions based on 50 μ l injected dose.

Wide distributions of pO_2 have been measured using ^{19}F MRI in diverse tumors in rats and mice (*e.g.*, Dunning rat prostate R3327-MATLu, -AT1, -HI and H, 13762NF rat breast tumor, human A549 and H460 lung tumors, Lewis lung carcinoma, mouse liver tumors, Shionogi tumors [83, 132, 219, 228, 230, 231, 233–238]) with hypoxic fractions ranging from $HF_{10} = 16\%$ in small R3327-H tumors to 83% in large R3327-AT1 tumors on anesthetized rats breathing air [134]. We have found repeat measurements are highly reproducible and generally quite stable under baseline conditions [236, 238–240], though Gallez *et al.* noted some fluctuations [228]. The ability to detect fluctuations is likely to be strongly influenced by spatial resolution, since larger volumes will exhibit damped responses. ^{19}F MR oximetry is particularly useful for evaluating acute response to interventions. The most common studies have involved hyperoxic gas breathing challenges, including comparison of oxygen and carbogen (CB) [173, 233, 235–243], since these are predicted to modulate tumor hypoxia, but there remains controversy regarding which is more effective as a potential adjuvant for radiation therapy. As shown in Fig. 4 baseline tumor oxygenation is generally heterogeneous, though quite stable. Upon hyperoxic gas breathing challenge, the well-oxygenated tumor regions responded rapidly and significantly with pO_2 increasing from 38 and 40 torr to 162 and 198 torr, respectively. One initially hypoxic voxel showed significant response increasing from 3 ± 2 to 14 ± 3 torr, while the other hypoxic region showed no change remaining below 2 torr. Other studies have examined androgen dependence via castration [219], vasoactive agents [239] and vascular disrupting agents (VDAs) [231, 244–246]. The study of arsenic trioxide by Gallez *et al.* is potentially paradigm shifting since VDAs are expected to cause vascular shutdown [247, 248] and consequent hypoxia, whereas ^{19}F MRI revealed elevated pO_2 at low doses likely associated with reduced oxygen consumption resulting from mitochondrial impairment [231]. Estimates of pO_2 and modulation of tumor hypoxia have been shown to be consistent with modified tumor response to irradiation [234, 241].

A crucial aspect has been validation of the measurements. Mason *et al.* demonstrated that both pO_2 and temperature in a perfused rat heart estimated by ^{19}F NMR T_1 relaxation of perfluorotributylamine matched independent estimates using oxygen (polarographic electrode) and temperature (fiber optic) probes [198]. Over the years diverse correlations have been reported, *e.g.*, pO_2 distributions found in small or large rat tumors when animals breathed air or oxygen were found to be similar by Eppendorf Histogram or ^{19}F NMR [249]. Local changes in pO_2 in response to breathing O_2 were remarkably similar by ^{19}F MRI or polarographic electrodes or fiber optic probes [240, 249–251]. Results are also consistent with hypoxia estimates using the histological marker pimonidazole [237]. Comparing identical tissue locations has not been feasible, but typical behavior was highly consistent. As an example, hypoxic regions in many tumors resist modulation with hyperoxic gas breathing, while well oxygenated regions show a large response (Fig. 4): matching data have been reported for ^{19}F NMR or electrodes [236, 243, 250]. Meanwhile, some tumors show a large response to hyperoxic gas breathing despite baseline hypoxia and again this was seen using ^{19}F MRI and fiber optic probes [240]. Gallez *et al.* compared two PFCs and found very similar baseline data for perfluoro-15-crown-5-ether and HFB in muscle [229]. However, 24 hrs later measurements no longer matched, which may be attributed to the differential clearance rates of the two PFCs. HFB typically clears with a 6

hr half-life from well-perfused tissues and there was little remaining after 24 hrs [188]. Since clearance of PFCs is believed to be via diffusion to the vasculature and first pass exhalation from the lungs [212, 252] it appears reasonable that any remaining HFB was in the less well perfused and presumably more hypoxic regions. Similarly, we normally find that insufficient HFB remains after 24 hrs for *FREDOM*. However, following administration of the VDA CA4P (Combretastatin, Zybrestat®), the vasculature shutdown was accompanied by rapid hypoxiation. On this occasion HFB was still detectable the next day, allowing further oximetry and observation of reoxygenation for some regions [245].

Further validation was recently provided by Baete *et al.* based on phantom studies with HFB either suspended in a gelatin matrix or in the flowing component [253]. In the oxygen consuming phantom (including yeast) the ultimate pO_2 measured using PFC reporter closely matched the pO_2 expected in the absence of perfluorocarbons. Simulations and modeling suggested that PFC in the flowing component could substantially perturb the measured pO_2 [254]. The quality of ^{19}F pO_2 determined from R_1 measurements depends on SNR [176]. Higher signal is associated with better curve fits and less uncertainty. This may be achieved by increasing data acquisition times (at the expense of temporal resolution), increasing voxel sizes (at the expense of spatial resolution) or administering more HFB (more invasive).

HFB is reported to be non-toxic [255–257] and it was considered as a veterinary anesthetic [258], though abandoned due to its low flash point in the gaseous phase [259]. When studying rats we now pre-anesthetize the rats and then turn off the isoflurane flow for some time during the injection of HFB, in order to avoid hemodynamic stress, which appears to recover within a few minutes [173, 227].

In addition to systemic administration of PFC emulsion or direct injection into a tissue of interest, it may be loaded into cells in culture prior to placement in an animal. This method is being developed primarily for tracking stem cell migration and retention, but also allows oximetry [6]. Initially the approach provides optimal signal to noise from the cells and assuredly provides intracellular measurements, though following cell division the density of fluorine per cell is reduced and cell death will lead to redistribution potentially to macrophages. An earlier version of this approach embedded cells and PFC in a polyalginate matrix, hence reporting changes close to cells [260, 261]. Placing spectrally separate PFCs intracellularly and in the local environment allows both oxygen delivery and local concentration to be assessed simultaneously [262].

Any technique has optimal applications and potential limitations. Currently, ^{19}F is not usually possible on human scanners and thus alternative approaches may be better suited to translational oximetry. Recently, we demonstrated an analogous proton MRI approach for oximetry in small animals based on hexamethyldisiloxane (HMDSO), which exhibits many properties closely similar to PFCs: notably high gas solubility, single resonance which is highly responsive to pO_2 [225, 226, 263]. It has the drawback of requiring water suppression, but this was effectively implemented and is particularly convenient, since HMDSO co-resonates with tetramethylsilane (TMS), the universal 1H NMR standard. Ultimately, it would be preferable to avoid the need for an exogenous reporter molecule and an increasing number of studies have indicated that T_2^* (BOLD) and T_1 (TOLD) of tissue water are sensitive to tissue oxygenation based on [dHb] and $[O_2]$ [79, 83, 264]. Correlations have been shown between BOLD and independent oximetry, though they are not always directly related [83, 216, 265]. Thus, quantitative oximetry based on ^{19}F MR may be most useful for calibrating the less invasive methods in pre-clinical investigations. To date HFB remains the most sensitive PFC to changes in pO_2 (η - slope/intercept), the least sensitive to temperature interference and is cheap and readily available (Table 3).

3.2 Detection of ions: metals and pH

Ions are generally detected based on pairing with a reporter molecule which undergoes a binding-dependant chemical shift. The earliest example of a metal ion reporter was probably 5F-BAPTA (5,5-difluoro-1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) (Fig. 1, Table 1), which shows $\Delta\delta \sim 6.0$ ppm chemical shift upon binding Ca^{2+} [117]. Metal ion binding is often in the slow exchange regime, so that separate signals are seen for the free and metal ion bound moieties, with chemical shifts of several ppm. Reporter molecules are generally designed to exhibit high specificity for the metal ion of interest, though 5F-BAPTA binds several divalent metal ions including Ca^{2+} , Zn^{2+} , Pb^{2+} , Fe^{2+} , and Mn^{2+} [117, 266]. In an averaged fast exchange signal regime this would cause interference, but here each metal ion complex has an individual chemical shift, so that they can be detected simultaneously [267]. Ion concentrations are estimated based on the signal ratio, avoiding the need for a chemical shift reference. The dissociation constant (K_D) does depend on pH, ionic strength, and free $[\text{Mg}^{2+}]$, which need to be estimated independently. 5F-BAPTA has been used to measure $[\text{Ca}^{2+}]$ in cell cultures [117, 268, 269], perfused tissue slices [270–273] and the perfused beating heart, revealing calcium transients during the myocardial cycle [274–276]. The two equivalent fluorine nuclei exhibit a single signal with enhanced SNR. Various isomers were also evaluated including 4F-BAPTA, which has a somewhat lower binding constant resulting in fast exchange and a single averaged signal from bound and unbound forms [267].

Fluorinated NMR reporters have also been demonstrated for $[\text{Mg}^{2+}]$ and $[\text{Na}^+]$ [276–280] and tested in biological systems including multiparametric assessment of Ca^{2+} , Mg^{2+} and pH in perfused ferret hearts [276]. Many other metal ion binding fluorinated ligands have been reported with rings optimized to accommodate the metal ion of interest, notably series of cyclams and cryptands, though these do not appear to have been used *in vivo* [281, 282] (Table 1). Jiang *et al.* [283] have demonstrated a tris trifluoromethyl Gd-based chelator which may be sensitive to as many as 20 different metal ions, based on differential chemical shift, and T_1 and T_2 relaxation, and they suggest the possibility of “multi-chromic” F-19 imaging tracers based on pairing metal ions with a fluorinated chelator.

pH sensitive ^{19}F NMR indicators were pioneered by Deutsch *et al.*, notably exploiting a series of fluoroalanines to investigate intra- and extracellular pH [116, 284–288]. On the NMR time scale, protonated and deprotonated moieties are generally in fast exchange, so that a single signal is observed representing the amplitude weighted mean of acid and base forms. The early studies focused on cell cultures with a goal of measuring transmembrane pH gradients. However, reporter molecules often failed to penetrate cells and thus cell penetrating pro-drugs were developed based on labile esters, which are cleaved by non-specific intracellular esterases. There is a fine balance between esters being too labile with consequent extracellular degradation versus resistance to cleavage, so that they fail to liberate intracellularly. The same issue applies to many chemotherapeutic drugs. Pro-drugs may be washed away in culture, but *in vivo* complex spectra may result from overlapping multi-line ester and liberated free acid resonances from both intra- and extracellular compartments [116].

The fluoroalanines have a relatively small chemical shift range (~ 2 ppm), which is quite typical of aliphatic indicators. In contrast aromatic reporter molecules can have a much larger chemical shift response [106, 127, 289], which has been exploited for measuring pH and by analogy enzyme activity described in Section 4 (Tables 1, 2 and 4). The vitamin B6 analogue fluoropyridoxol had been used to investigate activity of phosphorylase enzymes [290] and we adopted this molecule for measurements of pH in cells and perfused organs [17, 106, 289]. 6-FPOL readily penetrated red blood cells so that both intra- and extracellular pH (pHi and pHe) could be measured simultaneously in whole blood [289].

Measurements were also readily performed in perfused rat heart [17, 291]. However, FPOL does not appear to enter most tumor cells. Extensive penetration was found in a Morris hepatoma cell line transfected to express thymidine kinase [107]. The differential entry into blood cells may be related to facilitated transport, since vitamin B6 is naturally stored, transported, and redistributed by erythrocytes [292]. The fluoride ion itself has been proposed as a pH reporter exhibiting $\Delta\delta = 42$ ppm between pH 2 and 4 [293].

To enhance SNR a pH-sensitive CF_3 moiety was introduced in place of the F-atom, but the chemical shift response of 6-trifluoromethylpyridoxol (CF_3POL) was found to be much smaller (Table 2), as expected since electronic sensing must be transmitted through an additional C-C bond [111]. The pK_a was found to be better suited to normal tissue physiology and tumor ($\text{pK}_a \sim 6.8$ vs. 7.4), but the water solubility was lower confounding attempts to achieve higher SNR. Water solubility can be enhanced by adding sugar residues though this was found to increase the pK_a and was not pursued further. CF_3POL occurred exclusively in the extracellular compartment [107, 294]. While this confounds the ability to directly assess transmembrane pH gradients it has the potential advantage of defining which compartment is being observed. Since tumors are generally heterogeneous with a wide range in pH, the consequent broad lines representing intracellular pH (pH_i) and extracellular pH (pH_e) may not be resolved.

Most pH measurements have been limited to a global assessment, although *p*-fluorophenols were successfully used to map pH in a multi-vial phantom [103] due to the large chemical shift response ($\Delta\delta$ 6.4 to 9.3 ppm) to pH [295]. However, fluorophenols may act as ionophores, by analogy with dinitrophenol restricting their applicability *in vivo*. Molecular structure is crucial: *o*-fluorophenols have a smaller chemical shift range (~ 0.3 to 2.2 ppm) and meta orientation is even less sensitive [111]. A fluoroaniline sulphonamide (ZK150471) was used to measure tumor pH in mice and rats [296–299] and it is also restricted to the extracellular component. Combining with ^{31}P NMR of inorganic phosphate (P_i) to determine pH_i revealed a transmembrane pH gradient in mouse tumors [46], but a distinct problem with ZK150471 is that the pK_a differs in saline and plasma [298].

We have generally used sodium trifluoroacetate, as a non-titrating chemical shift reference, but an intramolecular chemical shift reference is feasible as demonstrated, with NEAP [300], 6-FPOL-5- α - CF_3 [291] and ZK150471 [299].

It should be recognized that the ^{31}P NMR chemical shift sensitivity of P_i has been widely used for measuring tissue pH for many years, but there are shortcomings motivating the search for more sensitive approaches. In healthy tissue P_i may occur at too low concentrations for effective measurements, but diseases such as ischemic insult generate extensive P_i [301]. Tumors tend to be somewhat hypoxic and exhibit extensive glycolysis, so that there is usually extensive P_i and separate signals attributable to intra- and extracellular compartments were fundamental to the recognition of reversed pH gradient characteristic of tumors [302]. Nonetheless, tumor signals are often broad and intra- and extracellular P_i are often only partially resolved due to the small chemical shift response ($\Delta\delta \sim 2.4$ ppm) [93].

Pioneering studies of fluorinated reporters for pH and metal ions stimulated the field of ^{19}F NMR molecular reporter agents. To date most studies have been limited to perfused cells and organs with global spectroscopic measurements. Nonetheless, much has been learnt regarding molecular design in terms of sensitivity, specificity, and cellular/membrane transport.

4 Proteomics

The most diverse recent innovations in ^{19}F NMR have applied to detection of proteins. In most cases detection was based on chemical shift response to enzyme mediated cleavage of a reporter substrate. Further innovations have exploited differential ^{19}F visibility due to paramagnetic relaxation enhancement (PRE) or restricted molecular mobility. Diverse reporter substrates, protein/enzyme targets, and references are presented in Table 4.

^{19}F NMR has long been applied to evaluating pharmacology in terms of catabolic and anabolic conversions of drugs and pro-drugs, as described in Section 2 and reviewed by others [29, 38, 303]. Notably, the popular chemotherapeutic 5-FU is converted to multiple products such as 5-fluoronucleotides, 5,6-dihydrofluorouracil, and α -fluoro β -alanine and the relative processes may influence therapeutic efficacy. ^{19}F NMR was also applied to 5-FC developed to be a less toxic pro-drug in combination with gene therapy and expression of cytosine deaminase (CD). Stegman *et al.* first showed the 1.5 ppm chemical shift in transfected cells infused with 5-FC into the peritoneum of mice [51]. Following proof of principle this approach was applied to solid tumors growing in rats and mice revealing enzyme activity [52, 53, 304]. Equally pertinent can be the observation of pro-drug with no metabolism indicating lack of gene expression [107].

The reports of ^{19}F NMR assay of CD activity together with elegant proton MRI of β -gal activity [305] prompted us to explore whether appropriate substrates could be developed to reveal β -gal activity by ^{19}F NMR. β -gal is reported to exhibit extremely broad substrate specificity (promiscuity) [305–311], but we chose a simple fluorinated analog of the traditional colorimetric yellow reporter ONPG (*o*-nitrophenylgalactoside) as an initial ^{19}F NMR active analogue [18]. Of note, fluorinated nitrophenol galactosides had been reported previously to explore β -gal activity [312]. However, they had placed a fluorine atom on the sugar moiety, which would be expected to provide little chemical shift response to cleavage, but was suitable for the ^{18}F PET investigations.

Our prototype molecule used a para fluoroaryl substituent in 4-fluoro-2-nitrophenyl β -*D*-galactopyranoside (PFONPG, Tables 2 and 4, Fig. 5) [18]. It has a single ^{19}F NMR signal with a narrow linewidth and good stability in solution, but cleavage of the glycosidic bond produced a substantial chemical shift $\Delta\delta > 3.6$ ppm. Initial tests were conducted in solution with β -gal enzyme, followed by transfected cells and ultimately stably transfected human tumor xenografts in mice [18, 19, 313]. Conversion reflected the level of enzyme expression with some *lacZ* tumors found to exhibit unexpectedly slow conversion validated by post mortem histology [313]. The substrate appeared stable in whole blood and wild type (WT) cells, although a low level background expression of β -gal is found in some cells [19, 311], which has been associated with senescence [314].

The chemical shift accompanying cleavage depends strongly on the orientation of the F-atom with largest response for para-F and less for ortho-F [295]. As expected [315], the rate of cleavage was found to depend on the pK_a of the aglycone (Fig. 6) [295]. To date our observations *in vivo* have required direct intra tumoral injection of substrates [19, 186, 313]. This has allowed us to correctly identify *lacZ* transfected tumors from wild type using a reporter pair 4-fluoro-2-nitrophenyl β -*D*-galactopyranoside (PFONPG) and 2-fluoro-4-nitrophenyl β -*D*-galactopyranoside (OFPNPG) in breast tumor xenografts in mice [313]. Each substrate and product has a unique chemical shift and thus multiple agents can be detected simultaneously. Separate resonances allowed two tumors to be assayed simultaneously, since each tumor had a different signal and therefore provided *de facto* local information without the need for imaging. In solution we have detected several reporters simultaneously (Fig. 5) and this approach could potentially facilitate *in vivo* proteomics.

Reporters could be designed each to be sensitive to a specific enzyme allowing simultaneous detection and we demonstrated proof of principle with respect to β -*D*-galactopyranosyl and β -*D*-glucosopyranosyl substrates and galactosidase and glucosidase [313, 316].

The fluoronitrophenol aglycone is potentially toxic and we have synthesized isomers and analogues designed to be less toxic. 3-*O*-(β -*D*-galactopyranosyl)-6-fluoropyridoxol (GFPOL) is less toxic, but was also found to be less reactive and less water soluble [317]. Water solubility and reactivity can be enhanced by polyglycosylation of the hydroxymethyl arms [316], as also discussed for potential pH indicators [294].

Generally imaging is much slower due to lack of signal, but we have observed β -gal induced cleavage of 2-fluoro-4-nitrophenol- β -*D*-galactopyranoside [318] and *p*-trifluoromethyl-*o*-nitrophenyl β -*D*-galactopyranoside (PCF₃ONPG) [111] in under 5 minutes and now show accelerated chemical shift selective echo planar imaging (EPI) in Fig. 6. A trifluoromethyl (CF₃) reporter group should enhance signal to noise, but as noted for pH indicators (Section 2), the chemical shift response is much smaller (Table 2), [111, 294].

Several substrates and target enzymes are shown in Table 4. In most cases the chemical shift response is larger when the reporter moiety is aromatic, as opposed to aliphatic, and fluoronitrophenols have been popular. Proximity of the signaling fluorine to the site of cleavage is also critical, although in some cases a self-immolative cascade allows greater spacing [109, 166, 187].

Noting that ¹H MRI provides much greater signal, we recently demonstrated detection of β -gal using T₂*-weighted MRI of S-Gal[®] [319], as well as several analogs providing enhanced T₁ and T₂* contrast [320]. A potential problem with proton MRI is differentiating enzyme/reporter indicator contrast from the natural heterogeneity of tumors. Thus, we designed dual ¹H/¹⁹F reporters, whereby the ¹⁹F signal should verify the presence of the reporter, while ¹H contrast provides spatially resolved evidence [186]. Our first generation agents, fluorobenzaldehyde aroylhydrazones, yielded a 5.2 to 7.7 ppm chemical shift upon enzyme activated hydrolysis. In the presence of ferric ions a complex is formed, which also generates strong T₂* proton MRI contrast. However, the interaction with Fe³⁺ causes ¹⁹F signal disappearance [186]. At this stage we do not know whether signal loss was due to the proximity of the paramagnetic center or precipitation induced line broadening. A second generation agent uses a fluorocatechol aglycone and both substrate and product are visible by ¹⁹F NMR ($\Delta\delta$ 6.3ppm) together with Fe³⁺ dependant contrast [321]. We note others have specifically examined enzyme mediated fluorocatechol degradation by NMR [322–324]

Combined ¹H and ¹⁹F sensitivity was recently demonstrated to overcome a major historic problem associated with ¹H MRI contrast: specifically, how to separate the contributions of reporter agent concentration from parameter dependant relaxivity. Aime *et al.* [325] incorporated a ¹⁹F moiety, whereby its signal provided molecular quantitation to calibrate the supramolecular poly- β -cyclodextrin-¹⁹F-Gd pH-dependant ¹H MRI contrast. In vivo application may be handicapped by differential spatial resolution typical of ¹H and ¹⁹F MRI, but there is clear proof of principle. Multimodality approaches have also been presented for detection of alkaline phosphatase and proteases based on evolution of both fluorescence and ¹⁹F [165, 185].

Recent reports have demonstrated a PRE approach to detecting enzyme activity or ions [109, 163–166, 187, 326]. Substrates include a ¹⁹F moiety in close proximity to paramagnetic center exerting strong T₂* relaxation and rendering it “invisible”. Enzyme induced molecular cleavage separates the fluorine moiety from the relaxing center producing enhanced ¹⁹F signal and consequently revealing activity as demonstrated for caspase, β -gal [187, 326], and β -lactamase [327]. A gene reporter has been suggested using a stem-loop

structured oligodeoxyribonucleotide based on a sequence for point mutated K-ras mRNA [328]. A bis(trifluoromethyl)benzene moiety experiences strong line broadening from associated Gd-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid until the loop separates upon binding the target complementary mRNA. Mismatched mRNA did not elicit the signal enhancement. Tanabe *et al.* [329] have demonstrated reversible ^{19}F NMR signal regulation using a ferrocene modified silicon scaffold (a ferrocene moiety for signal modulation, trifluoroacetyl groups for the ^{19}F NMR signal, and cubic octameric polyhedral oligomeric silsesquioxanes (POSS) as a scaffold). ^{19}F NMR signal was modified by the valence state of the ferrocene moiety of the probe via PRE from the ferrocene iron (diamagnetic Fe^{2+} versus paramagnetic Fe^{3+}). Line broadening and signal enhancement are relative and judicious signal separation of the fluorine reporter and relaxing center has been used to enhance acquisition efficacy based on reduced T_1 , while avoiding excessive T_2^* line broadening [166].

NMR visibility is also influenced by molecular motion, whereby restricted motion causes line broadening, which is expected to be particularly strong due to the relatively large chemical shift anisotropy of ^{19}F . Several examples have now shown that disruption of aggregate nanostructures produces enhanced ^{19}F NMR signal [112, 154, 185, 330]. Judicious incorporation of protein ligands led to recognition driven disassembly of nanoprobe accompanied by strong signal gain. Examples were demonstrated with human carbonic anhydrase I (hCAI) detection using a probe consisting of three modules: (i) a hydrophilic ligand specific to a protein of interest (head group, benzenesulfonamide for hCAI), (ii) a hydrophobic 3,5-bis-(trifluoromethyl)benzene for the ^{19}F NMR detection modality (tail group), and (iii) a relatively hydrophobic linker group to connect these two modules. Other examples were shown with biotin-tethered probes used to detect avidin and methotrexate (MTX) as a specific probe of dihydrofolate reductase (DHFR) [112, 154]. The concept has been explored in some detail yielding molecular complexes, which can be tailored to be “always on”, “turned on” or “turned off” [112]. Restricted motion modulating linewidth was demonstrated as a potential key to molecular recognition using a ^{19}F -based lectin biosensor for selective detection of glycoproteins. [331].

The ^{19}F NMR reporters most widely used *in vivo* have been fluoronitroimidazoles to detect hypoxia (Tables 1 and 2). These undergo multistep nitroreductase activity generating highly reactive species, which are trapped in the absence of oxygen [132]. This approach is widely applied to radiochemical substrates, such as ^{18}F -misonidazole and ^{18}F -EF5 for use with PET, which detects all labeled molecules irrespective of molecular alterations. For NMR the diverse adducts, and metabolites are expected to exhibit multiple chemical shifts, each at very low concentration and polymeric adducts could have exceedingly short T_2 , rendering them invisible [332, 333]. Nonetheless, several NMR studies have shown ^{19}F spectra in pre-clinical animal and human patient tumors [119, 133, 136–139, 334, 335]. Indeed, Procissi *et al.* [140] recently demonstrated heterogeneous uptake of a trifluoromethylated nitroimidazole using ^{19}F MRI. Some reports have noted potential discrepancies in uptake of fluoronitroimidazoles potentially attributed to levels of nitroreductase expression, perfusion, blood flow and glutathione concentration [133, 334, 335] independent of hypoxia [133].

In other cases ^{19}F NMR assays are simply based on molecular accumulation. A series of fluoroheptuloses was recently presented to assay GLUT2 activity based on insulin dependent uptake and trapping in pancreatic cells [336]. ^{18}F -FDG PET [132] has become a routine test for tumor metastasis in patients based on phosphorylation induced trapping in metabolically active cells. However, ^{19}F NMR investigations require much higher concentrations and different metabolic pathways may dominate: 2-F and 3-F isomers have been examined *in vivo* [337–340]. Accumulation of (E,E)-1-fluoro-2,5-bis(3-hydroxycarbonyl-4-hydroxy)styrylbenzene (FSB), has been demonstrated in brains of APP

transgenic mice known to develop amyloid plaques [341]. While ^{19}F imaging required about 2 hrs, accumulation was observed *in vivo* following IV infusion of the reporter. Amatsubo *et al.* [342] attempted to increase SNR by introducing a CF_3 group in place of a fluorine atom. However, they report that ^{19}F signal was lost upon binding to plaque, which they attribute to line broadening from restricted motion of the more lipophilic agent. A more recent example used a ^{19}F -containing curcumin derivative, 1,7-bis(4'-hydroxy-3'-trifluoromethoxyphenyl)-4-methoxycarbonyl-1,6-heptadiene-3,5-dione to detect amyloid in brains of Tg2576 mice [343].

We have focused on the design of reporter molecules tailored to specific enzymes. Potentially, multiple reporters may be applied simultaneously revealing individual activities. This is particularly appropriate for ^{19}F NMR given the large chemical shift range. We note that several molecular structures have been described for the more popular enzymes such as β -gal and nitroreductase. However, a separate molecule must be designed and synthesized for each enzyme. Moreover, different leaving groups may exhibit differential reaction rates (Fig. 5). An alternative approach has been suggested for potential high throughput screening of multiple enzymes: notably use of a common substrate. ATP is required for many reactions, particularly kinases, which regulate many pathways. 2'-fluoro-ATP failed for nicotinamide adenine dinucleotide synthetase, but did function with 3-phosphoinositide dependent kinase 1 (PDK1) with $\Delta\delta \sim 0.4$ ppm upon conversion to 2-fluoro-AMP indicating substrate selectivity. Substrate modification to 2-fluoro-ATP provided successful investigation of nine enzymes representative of three enzyme subclasses: transferase, hydrolase, and ligase. While the chemical shift between 2F-ATP and 2F-AMP is only about 0.2 ppm, it allowed *in vitro* screening of enzyme inhibitors [344]. When the product was 2-fluoro-ADP change was barely detectable with overlapping signals.

5 Conclusion

^{19}F NMR has been developed to probe many diverse physiological, pharmacological, and metabolic parameters. The chemical shift and relaxation rates are particularly sensitive to the molecular environment. Not only can ^{19}F NMR offer unique insights, but the inherently quantitative nature of NMR is attractive. A major problem with proton MRI contrast is inherent tissue heterogeneity so that reporter molecule responses may not be obvious. ^{19}F can be definitive and promises many applications for molecular biology, pre-clinical small animal *in vivo* tests, and ultimately human investigations.

Acknowledgments

Supported in part by NCI 1U24CA126608, P30CA142543, P41RR02584 1R21CA120774, 1R01CA125033, and 1R01CA139043.

Glossary of Abbreviations

15C5	Perfluoro-15-crown-5-ether
5-FC	5-Fluorocytosine
5-FU	5-Fluorouracil
6-FPOL	2-Fluoro-5-hydroxy-6-methyl-3,4-pyridinedimethanol
ADMET	Absorption, Distribution, Metabolism, Excretion, and Toxicity
ATP	Adenosine triphosphate
BAPTA	1,2-bis(<i>o</i> -amino-phenoxy)ethane- $\text{N},\text{N},\text{N}',\text{N}'$ -tetraacetic acid

BESR	Blood Enhanced Saturation Recovery Sequence
BOLD	Blood Oxygen Level Dependent
CB	Carbogen
CD	Cytosine Deaminase
DHFR	Dihydrofolate reductase
DHFU	5,6 Dihydrofluorouracil
DMSO	Dimethyl sulfoxide
DTPA	Diethylenetriaminepentaacetic acid
EF5	2-(2-nitro-(1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-acetamide
EPI	Echo Planar Imaging
FLASH MRI	Fast Low Angle SHot magnetic resonance imaging
FNuct	5-fluoronucleotides
FRET	Fluorescence (Förster) Resonance Energy Transfer
FBAL	α -Fluoro β -alanine
FDG	Fluorodeoxyglucose
FREDOM	Fluorocarbon Relaxometry using Echo planar imaging for Dynamic Oxygen Mapping
FSB	(E,E)-1-fluoro-2,5-bis(3-hydroxycarbonyl-4-hydroxy)styrylbenzene
GFPOL	3- <i>O</i> -(β - <i>D</i> -galactopyranosyl)-6-fluoropyridoxol
Glut2	Glucose transporter type 2
HFB	Hexafluorobenzene
HMDSO	Hexamethyldisiloxane
IUPAC	International Union of Pure and Applied Chemistry
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
MTX	Methotrexate
NaTFA	Sodium Trifluoroacetate
NEAP	N-Ethylaminophenol
NMR	Nuclear Magnetic Resonance
ONPG	O-Nitrophenylgalactoside
OFFNPG	2-fluoro-4-nitrophenyl β - <i>D</i> -galactopyranoside
PCr	Phosphocreatine
PET	Positron emission tomography
PCF₃ONPG	<i>p</i> -Trifluoromethyl- <i>o</i> -nitrophenyl β - <i>D</i> -galactopyranoside
PDK1	3-phosphoinositide dependent kinase 1
PFC	Perfluorocarbon

PFOB	Perfluorooctyl bromide
PFONP	4-Fluoro-2-nitrophenol
PFONPG	4-Fluoro-2-nitrophenyl- β -D-galactopyranoside
PFTB	Perfluorotributylamine
pHe	Extracellular pH
pHi	Intracellular pH
Pi	Inorganic Phosphate
pO₂	Partial Pressure of Oxygen
POSS	Polyhedral oligomeric silsesquioxane nanoparticles
PRE	Paramagnetic relaxation enhancement
RES	Reticuloendothelial system
SF₅	Pentafluorosulphonyl
SI	Signal intensity
SNR	Signal to Noise Ratio
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
TGI	Total global ischemia
TMS	Tetramethylsilane
TOLD	Tissue Oxygen Level Dependent
VHL	Von Hippel-Lindau

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Figure 1. Representative fluorine-based reporter molecules

Increasing the number of equivalent fluorine atoms should enhance signal to noise and efficacy of reporter molecules: diverse reporters are presented with 1 to 20 equivalent fluorines, although the SF_5 group is an AX_4 spin system providing two separate signals. i) 3-fluoro-2-methyl alanine, ii) 3,3-difluoro-2-methyl alanine, iii) 3,3,3-trifluoro-2-methyl alanine, iv) PFONP: 4-fluoro-2-nitrophenol, v) 5F-BAPTA: 5,5-difluoro-1,2-bis(*o*-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid, vi) PCF₃ONP: 4-trifluoro-2-nitrophenol, vii) 4-hydroxyphenylsulfurpentafluoride, viii) HFB: hexafluorobenzene, ix) CCI-103F, x) 5-methoxy-1,2-dimethyl-3-[[2,2,2-trifluoro-1,1-bis(trifluoromethyl)ethoxy] methyl]-1H-

indole-4,7-dione, xi) dimeric 3,5-bis-(trifluoromethyl)benzenyl moiety, xii) 15C5:
perfluoro-15-crown-5-ether.

Figure 2. Recognition-driven disassembly of the nanoprobe with enhanced ^{19}F NMR/MRI for additional fluorine atoms

Takaoka *et al.* [112] developed a method for specific protein detection based on recognition-driven disassembly of fluorinated “nanoprobes”. A) The nanoprobe is a supramolecular aggregate of fluoroaryl reporter moieties tightly packed with protein recognition ligands. When packed, the restricted molecular motion causes strong line broadening. Upon interaction with the target protein the aggregates disassemble accompanied by ^{19}F signal narrowing and detectability. B) Various amphiphilic fluoroaryl esters were used tailoring the head group to interact with the target protein, the linker to modulate stability and the tail to provide responsive ^{19}F signal. C) The signal to noise was found to increase with additional fluorine atoms: ^{19}F images were obtained at 282 MHz and are shown for ArF vs. bis $(\text{CF}_3)_2$ groups in the presence of hCAI. Relative integrals are shown in graph. Adapted with permission from Takaoka *et al*, *J. Am. Chem. Soc.*, **2011**, *133* (30), 11725–11731. Copyright (2011) American Chemical Society.

Figure 3. Localization of labeled cells after *in situ* injection

A) To determine the utility for cell tracking stem/progenitor cells labeled with either PFOB (green) or perfluoro-15-crown-5-ether (CE) (red), nanoparticles were locally injected into mouse thigh skeletal muscle. B–D, At 11.7 T, spectral discrimination permitted selective imaging of ¹⁹F signal attributable to PFOB-loaded (B) or 15C5-loaded cells (C) individually, which when overlaid onto a conventional ¹H-image of the site (D) revealed PFOB-labeled and CE-labeled cells localized to the left and right leg, respectively (dashed line indicates 3.3 cm² field of view for ¹⁹F-images). E, F, Similarly, at 1.5 T, ¹⁹F-image of $\sim 4 \times 10^6$ CE-loaded cells (E) locates to the mouse thigh in a ¹H-image of the mouse cross section (F). The absence of background signal in ¹⁹F-images (B, C, E) enables unambiguous localization of perfluorocarbon-containing cells at both 11.7 T and 1.5 T. (Reprinted with permission from Partlow KC *et al.* [10]. Copyright FASEB)

Figure 4. Tumor oximetry using ^{19}F MRI

Dunning prostate R3327-AT1 tumor was implanted in rat thigh and allowed to grow to about 9 mm diameter. Images show pO_2 maps derived using *FREDOM*, while anesthetized rat breathed air and oxygen, following injection of 50 μl HFB directly into the tumor. pO_2 maps during air and oxygen breathing overlaid on a high resolution T_2W image (M muscle, B bone marrow, T tumor). Upper graph shows dynamic changes in pO_2 for four specific voxels (indicated by arrows) upon oxygen breathing. Locations exhibiting higher baseline pO_2 , e.g., voxels 1 and 2 ($\circ$, $\bullet$), show greater and more rapid response to oxygen-breathing challenge. Voxels 3 and 4 ($\square$, $\blacksquare$) show less response, although changes were significant for voxels 1–3 ($p < 0.01$).

Figure 5. ^{19}F NMR to detect multiple enzymes simultaneously

^{19}F NMR at spectra 400 MHz of three β -D-galactopyranosides PFONPG (red), OFPNPG (green), and OFPG (blue), in PBS at 37 °C with sodium TFA chemical shift reference (bottom) and following addition of β -gal (middle) 2 min and (upper) 34 min. A Brønsted plot of substrate susceptibility to β -gal (E801A, pH 7.4) versus pKa of liberated aglycone is shown in the upper panel (PFONP (■), OFPNPG (●), and OFPG (▲)). Adapted with permission from Yu *et al*, *Bioconj. Chem.*, 2004. **15**(6): 1334–1341. Copyright (2004) American Chemical Society [295].

Figure 6. ^{19}F NMR detection of β -gal expression using RIFLE (Rapid Imaging of Fluorinated Ligands for Enzyme activity)

^{19}F NMR at 188.2 MHz revealed conversion of OFPNPG to OFPNP upon addition of β -gal. A vial contained 2 mg TFA and 7.8 mg OFPNPG in 0.5 ml PBS and 1 unit β -gal was added. Alternated ^{19}F spectra and chemical shift selective EPI were performed showing decrease of OFPNPG over 30 mins accompanied by appearance of new product aglycone signal 59.6 ppm upfield. Serial spectra are shown on the left and chemical shift selective images on the right. The variations in signal intensity of substrate, product, and TFA standard are shown in the graph in the lower panel. Image acquisition time 33 s per resonance (data acquired in collaboration with Dr. Vikram Kodibagkar, currently at ASU).

Figure 7. Design Principle of the PRE-Based ^{19}F MRI Probe to Detect Protease Activity
Concept for activation of reporter molecule by enzyme induced cleavage of substrate to separate ^{19}F reporter moiety from paramagnetic relaxing center. A typical substrate structure is shown in Table 4: Gd-DOTA-DEVD-AFC. Reproduced by permission from Mizukami, *et al.*; *J. Am. Chem. Soc.* **2008**, 130, 794–795. Copyright (2008) American Chemical Society.

Table 1

Molecular reporter strategies based on ^{19}F NMR

Parameter Measured	Reporter Molecule	NMR Parameter	Representative applications and references
O_2	Perfluorocarbons <i>e.g.</i> , hexafluorobenzene (HFB), 15-crown-5-ether (15C5) perfluorooctyl bromide (PFOB) (perfluorotributylamine) PFTB	R_1 or occasionally R_2	Reviews: [105, 133, 173] Solutions, Phantoms and Theory: [170, 188, 195, 196, 227, 253, 345–350] In vivo: Mouse [122, 174, 176, 190, 211, 213, 219, 228–231, 351] Rat [4, 6, 83, 188, 205, 206, 208–210, 217, 233–242, 245, 249, 250, 261, 352–358] Rabbit [214, 216, 221, 359–363] Cat [197] Pig [170, 194, 364–366] Human [367]
Temperature	PFTB, PFOB	ΔR_1 or $\Delta\delta$ or signal ratio	[194, 198, 363]- perfused heart and tumor
pH	FPOL, CF_3POL , F-alanines, ZK150471 DFMO FQuene 5Fu metabolites	$\Delta\delta$, J	Reviews: [106, 288, 296, 299, 368] Applications: [17, 107, 116, 127, 291, 296, 298–300, 369–371] Cells, perfused organs: heart; tumor
$[\text{Ca}^{2+}]$ $[\text{Mg}^{2+}]$ $[\text{Na}^+]$	F-BAPTA F-APTRA F-cryp-1	δ , ratio	[117, 267, 270–272, 274–276, 372, 373] [278] [279, 280]
Other metal ions, <i>e.g.</i> , $[\text{Ni}^{2+}]$ $[\text{Cu}^{2+}]$ $[\text{Sr}^{2+}]$ $[\text{Ba}^{2+}]$ $[\text{Li}^+]$ $[\text{K}^+]$ $[\text{Rb}^+]$	F-Cyclam F-Dioxocyclam various ring sizes optimized to accommodate metal ion FN_2O_3 , FN_2O_4 F_2 -[2.1.1]-Cryptand + expansions 5F-BAPTA-many additional ions-each with unique δ	δ , or signal ratio	[266, 281–283, 374–376]
Hypoxia	F-nitroimidazoles	Signal integrals	[119, 132, 136–138, 140, 377, 378] Tumors in dog, human, mouse, rat
Gene Reporters, <i>e.g.</i> , <i>lacZ</i> and β -gal	PFONPG See also Table 4	$\Delta\delta$	[18, 19, 186, 295, 313, 316–318, 326] <i>In vitro</i> , <i>in cellulo</i> , mouse tumors <i>in vivo</i>

Table 2

^{19}F NMR Signal Enhancement: spectral and molecular characteristics of reporter molecules designed to detect pH, enzyme activity, and hypoxia exploiting varying numbers of F-atoms.

Reporter Structure	Multiplicity	pK_a	$\Delta \delta$ (ppm) ^I response	References and Applications
3-fluoro-2-methyl alanine	F $J_{\text{F,H}}$ coupling	8.5	Acid-base 2.05	[116, 284, 285, 287, 288, 347, 379] cells
3,3-difluoro-2-methyl alanine	2F ABQ and $J_{\text{F,H}}$ coupling	7.3	Acid-base 2.00	[116] cells
3,3,3-trifluoro-2-methyl alanine	3F Singlet	5.9	Acid-base 2.10	[116] cells
6-FPOL	F Singlet	8.2	Acid-base 9.72	[17, 106, 107, 289, 291] cells, perfused heart
CF ₃ POL	3F Singlet	6.8	Acid-base 1.7	[107, 294] cells, perfused heart
PFONP	F Singlet	6.9	Acid-base 9.3 ppm	[295]

Reporter Structure	Multiplicity	pK_a	$\Delta \delta$ (ppm) ¹ response	References and Applications
<p>PCF₃ONP</p>	3F Singlet	5.6	Acid-base 1.0 ppm	[111]
<p>PFONPG</p>	F Singlet	Rate ² 19 μ mol/min/ unit β -gal	Aglycone release 9.84ppm	[18, 19, 111, 295, 313] cells, tumors
3F Singlet	Rate ² 33 μ mol/min/ unit β -gal	Aglycone release 1.14ppm	[111] cells
<p>RO 07-0741</p>	F J _{H,F}	Partition coefficient ³ 0.44	Accumulation	[138, 380, 381] mice, tumors, cells
<p>SR 4554</p>	3F Singlet	Partition coefficient ³ 0.634	Accumulation	[133, 136, 137, 332, 335, 382, 383] tumors, man
<p>CCI 103F</p>	6F Singlet	Partition coefficient ³ 20	Accumulation	[119, 380, 384] mice, tumors

¹Chemical shift response to acid base titration ($\Delta \delta$ (ppm))

²Rate of enzyme activated hydrolysis (β -gal G-2513, 11 U) at 37 °C in PBS buffer (0.1 M, pH 7.4), (μ mol/min/unit β -gal)

³P_{Oct}; Octanol:water partition coefficient:

Table 3

Properties of Perfluorocarbon pO₂ Reporters

Perfluorocarbon (MW)	Resonances	F/mg [†]	F/μl [†]	Calibration curve R ₁ =A+B.pO ₂ ^{††}	Sensitivity η=slope/intercept	Temperature Sensitivity (sec. °C) ⁻¹	References [173]
PFTB (671)	4	0.013	0.024	A = 0.8848 B = 0.00226	2.6×10 ⁻³	0.021	7 T [198] 4.7 T [221]
PFOB (498)	8	0.006	0.011	A = 0.290 B = 0.00227	7.8×10 ⁻³	0.0059	7 T [199] 4.7 T [176]
15C5 (580)	1	0.034	0.063	A = 0.542 B = 0.0036	6.6×10 ⁻³	0.0094 [358]	7 T [6] 4.7 T, 9.4 T [354] 11.7 T [229]
HFB (186)	1	0.032	0.065	A = 0.102 B = 0.001658	16×10 ⁻³	0.0012	7 T [188, 227] 11.7 T [229]

PFTB: perfluorotributylamine, PFOB: perfluorooctyl bromide or perflubron (USAN), 15C5: 15-crown-5- ether, HFB: hexafluorobenzene.

[†] relative intensity of most intense ¹⁹F signal

^{††} Calibration curve R₁ (s⁻¹) = A + B.pO₂ (torr) at 7 T and 37 °C

Table 4

¹⁹F NMR Enzyme Sensitive Reporters

Gene or enzyme detected	Structures	Mode of action and MR response	References and applications
Method: chemical shift			
<i>Hydrolases e.g., lacZβ-galactosidase</i>		Release of aglycone Δδ 9.84 ppm: isomer dependent Δδ 5.2 to 7.7 ppm	Nitrophenol aglycones [18, 19, 295, 313, 316–318] <i>In vitro, in cellulose and in vivo</i> - see also Figs. 5, 6 [186] <i>In vitro</i> ,
Acid phosphatase Alkaline phosphatase		Release of phosphate	[385] [103] Enzyme in solution

Gene or enzyme detected	Structures	Mode of action and MR response	References and applications
Carboxypeptidase G2	<div><p>{4-[bis(2-hydroxyethyl)amino]-3,5-difluorobenzoyl}-L-glutamic acid 3,5-difluorobenzoyl-L-glutamic acid</p> </div>	<p>$\Delta\delta$ 5.8 and 8.8 ppm for separate resonances.</p> <p>$\Delta\delta$ 1.1 ppm</p>	<p>[386, 387] Cells transfected to express CPG2</p>
α -chymotrypsin		<p>$\Delta\delta$ 4.5 ppm</p>	<p>[166] <i>In vitro</i></p>
Cytosine deaminase		<p>$\Delta\delta$ 1.2 ppm</p>	<p>[51, 52, 54] Cells, tumors in mice and rats</p>
Galactose oxidase		<p>$\Delta\delta$ ~5 ppm</p>	<p>[388, 389] In CH₃CN to date</p>

Gene or enzyme detected	Structures	Mode of action and MR response	References and applications
Glucose phosphorylase	2-fluoro-2-deoxy- <i>D</i> -glucose (2-FDG) 3-fluoro-3-deoxy- <i>D</i> -glucose (3-FDG)	Multiple metabolite signals	[337, 338, 390, 391] [340, 392, 393] glycolytic and aldose reductase sorbitol (ARS) pathways mouse, rat, rabbit; brain, eye, tumor
Glutathione transferase		$\Delta\delta < 0.2\text{ppm}$	[394] <i>E.coli</i>
Histone deacetylase inhibitor (HDACi)	Boc-Lys-TFA-OH (BLT)	$\Delta\delta < 0.3\text{ ppm}$ release of TFA	[395, 396] tumor
Monoamine Oxidase Mao		$\Delta\delta\ 4.2\text{ ppm}$	[184] Enzyme <i>in vitro</i>

Gene or enzyme detected	Structures	Mode of action and MR response	References and applications
NADPH:cytochrome P450 reductase		$\Delta\delta$ 6 ppm	[329] A549 cells in culture
Method: Paramagnetic relaxation enhancement (PRE)			
<i>Hydrolases e.g., lacZ</i> β -galactosidase		$\Delta T_1, T_2^*$ Line broadening and visibility	[187, 326] <i>In vitro</i> See also Fig. 7
Caspase-3		$\sim 0.1 \Delta\delta$ ppm $\Delta T_1, T_2$	[164] <i>In vitro</i>
Signal visibility based on molecular mobility			
Alkaline phosphatase	Perfluorinated dendrimers anchored on silica nanoparticles	NMR visibility upon release required hours in cell lysate	[185, 330]
Glutathione reductase	Polyhedral oligomeric silsesquioxane (POSS) nanoparticles	NMR visibility upon release	[397] <i>In vitro</i> and cell lysates
Human carbonic anhydrase I (hCAI)	High molecular mass aggregates including fluorinated reporter moiety, <i>e.g.</i> , Fig. 2	NMR visibility upon release	[154]