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Review

The potential of nuclear magnetic resonance to track lipids in planta☆

Eberhard Munz [a, b], Peter M. Jakob [b, c], Ljudmilla Borisjuk [a, *]

[a] Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany
[b] Department of Experimental Physics 5, University of Würzburg, Würzburg, Germany
[c] MRB Research Center for Magnetic-Resonance-Bavaria, Würzburg, Germany

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ABSTRACT

Nuclear Magnetic Resonance (NMR) provides a highly flexible platform for non invasive analysis and imaging biological samples, since the manipulation of nuclear spin allows the tailoring of experiments to maximize the informativeness of the data. MRI is capable of visualizing a holistic picture of the lipid storage in living plant/seed. This review has sought to explain how the technology can be used to acquire functional and physiological data from plant samples, and how to exploit it to characterize lipid deposition *in vivo*. At the same time, we have referred to the current limitations of NMR technology as applied to plants, and in particular of the difficulty of transferring methodologies optimized for animal/medical subjects to plant ones. A forward look into likely developments in the field is included, anticipating its key future role in the study of living plant.

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Abbreviations: NMR, nuclear magnetic resonance; MRI, magnetic resonance Imaging; CSI, Chemical Shift Imaging; CSSI, Chemical Shift Selective Imaging; PRESS, Point-Resolved Spectroscopy; PFG, Pulsed Field Gradient; RF, Radio Frequency; MAS, Magic Angle Spinning; HSQC, Hetero-nuclear single quantum coherence; MALDI, matrix-assisted laser desorption/ionization; TD-NMR, Time-Domain NMR; TR, Repetition Time; TE, Echo-Time; SNR, Signal to Noise Ratio; MS, Mass Spectrometry; GC, Gas Chromatography.

☆ In memoriam of our former colleague Dr. Markus Rokitta. His creativity in plant NMR paved the way for current developments.

* Corresponding author.
E-mail address: borisjuk@ipk-gatersleben.de (L. Borisjuk).

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1. Introduction: where and why do plants accumulate lipids?

Through photosynthesis, plants convert light into chemical energy, in the form of the complex organic compounds carbohydrates, proteins and lipids. Carbohydrates are accumulated in the plant cell in the form of polysaccharides (in particular starch within the plastids, and cellulose in the cell wall), while storage proteins and lipids are typically compartmentalized within specialized inclusions, isolated from the cytoplasm by a single layer membrane [1–3]. The partitioning of fixed carbon between distinct metabolic pathways [4], cell compartments [5] and between plant organs [6] is a dynamic process, the regulation of which remains far from being resolved [7,8].

The fully reduced carbon bonds present in lipid molecules are associated with a higher energy density than the partially oxidized ones common in carbohydrate and protein molecules; this is because lipid molecules contain few carbon-oxygen bonds, making them weight for weight more energy dense than carbohydrates. Since a smaller volume is therefore required to store the equivalent amount of energy, many plants have evolved the capacity to store energy in the form of lipids, particularly in the seed. Most of the storage lipid content of plants exists in the form of triacylglycerides [9], although waxes are also encountered (for example crystalline wax *Myrica pensylvanica* (bayberry) [10], liquid wax in *Simmondsia chinensis* (jojoba) and others [11].

The proportion of a seed represented by lipid varies tremendously, but can reach as high as 70% in some nut species. Oils tend to be abundant only in the seed or fruit, but a few examples of other oil-rich organs have been documented, notably the tubers of *Cyperus esculentus* [12]. A ready source of available energy is particularly important for the germinating seed, supporting it until seedling has established its own photosynthetic apparatus.

2. The advantages of nuclear magnetic resonance (NMR) as an imaging and analytical tool

Plant “lipidomics” emerged with the initial aim to characterize lipids extracted from plant tissues, and has evolved into a broad-ranging study which comprises various aspects of lipid metabolism and functionality. Unfortunately, most of conventional techniques for tracking lipids rely on a destructive assay, which can only provide a static measurement of the lipid content of a specific set of cells or plant organs. The task of data integration appears to be limited, as evidenced in most of the performed studies, and the next challenge is to understand how these molecular, biochemical and structural characteristics work together in a living organism. In contrast, NMR offers a means to non-destructively (and therefore dynamically) assay lipids in living tissue, and thereby facilitates following the plant's growth and development, and its response to its exogenous environment.

Unlike the assays required for optical microscopy (where lipid detection is based on either staining or immuno-labeling), NMR does not require any labeling, nor does it entail complex sample preparation. In addition to detecting lipids, it can in principle also measure a number of physical and other chemical parameters, allowing an increasingly comprehensive picture of the *in vivo* status of the subject to be captured. A particularly powerful variant of NMR is magnetic resonance imaging (MRI), which, unlike X-rays, is based on the use of non-ionizing radiation, and is therefore non-harmful to the subject. In addition, MRI generates three dimensional images, thus overcoming the major drawbacks of both Fourier Transform Infrared Spectroscopy (FT IR) imaging and optical microscopy.

The capability of MRI to separate chemical compounds is much lower, as compared to gas chromatography [13], for ^{1}H-Lipid-Imaging it is restricted to the compounds shown in Fig. 1. Nevertheless, MRI is the only method which allows for both visualization and quantification of lipid in living plant.

3. The value of imaging of plant lipids *in vivo*

The need to develop the means to non-invasively detect lipids has been driven by the medical diagnostics sector, and has led to the elaboration of a number of NMR based protocols [14]. In the plant sciences, where destructive sampling has long dominated, advances in technology have concentrated on tissue dissection and extraction methodology. Although some of these analytical methods have achieved impressive levels of chemical resolution and sample throughput, reconstructing a living environment from data derived from destructive sampling can be extremely difficult or at best be based on a set of suggestions.

With the growing demand for oil, the modern requirements in plant biology and biotechnology are changing faster than ever before [15]. Various metabolic engineering strategies are developed for manipulation of oil content and composition in vegetative and seed tissues of plant [16,17]. The pressure to implement the non-invasive measurement of lipids in plants reflects progress in at least three disparate areas. The first relates to technical improvements in the productivity of established oil crops, in particular oil palm (*Elaeis guineensis*), soybean (*Glycine max*), oilseed rape

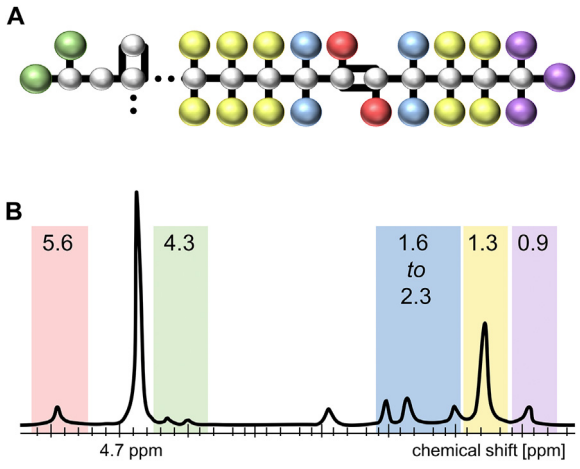


Fig. 1. Interpretation of 1H NMR spectrum. (**A**) Scheme shows fragment of lipid molecule made up from carbon (white) and hydrogen (variegated). (**B**) ^{1}H NMR spectrum (simplified) displays the signals acquired from protons of shown lipid molecule. The NMR frequency of the signal of protons varied corresponding to their molecular environment, as shown by the same color-code. Red: –**H**C=C**H**–; green: glycerol backbone; blue: $(CH_2)_n$–C**H**$_2$–HC=CH, C**H**$_2$–COOH, C**H**$_2$–CH_2COOH; yellow: $(CH_2)_n$; purple: $(CH_2)_n$-C**H**$_3$; The water peak appears at 4.7 ppm. The intensity of the individual peaks is informative for quantitative interpretation of the spectrum.

(*Brassica napus*) and sunflower (*Helianthus annuus*), which will increasingly require a more detailed understanding to be obtained of how the accumulation of lipids is regulated. Generating such data is only possible if the metabolism and structure of living plant tissue is not disturbed by sampling. The second area relates to the promotion of a number of novel candidate plant species as lipid factories, targeting growing environments not used for arable crop production; these species include *Jatropha curcas*, which is highly drought tolerant, various algae which can be grown in a closed system [18], and species such as *Camelina sativa* which produce unusual lipid forms in their seed [19]. Finally, advances in molecular biology are creating opportunities to design entirely novel cropping systems: one option is to engineer plants to accumulate lipids in their vegetative tissue, rather than solely in their seed [20,21], while an alternative is to redirect assimilate from its deposition as starch (or protein) into the synthesis and deposition of lipids [22]. The success of these new strategies will require a holistic approach to be taken to the study of lipid synthesis and deposition, in particular one in which the key processes can be followed in living tissue. Such an approach requires non-invasive imaging techniques.

This review promotes the use of MRI as a non-invasive, as highly informative technology for tracking lipids *in planta*. The platform can accommodate a variety of assays, ranging from single voxel proton spectroscopy to multidimensional chemical shift imaging, and from rapid global analysis to the production of high resolution images. The NMR applications described exemplify some of the major breakthroughs made in imaging over recent years, and cover much of the current literature describing the measurement of lipids and the imaging of whole plants, fruits and seeds.

4. How NMR images are formed

MRI does not rely on the projection, reflection or refraction of visible light, which provides the basis for all optical methods; instead, it depends on the behavior of nuclear spins of compounds present in the sample interacting with magnetic field. Thus, the underlying principle of image acquisition differs fundamentally to what applies in optical methods.

To create an image, the sample is placed within a homogeneous magnetic field B_0. The stable magnetic field is generated by a superconducting coil cooled down by liquid helium (4.2 K). There is no optimum field strength for MR imaging of lipid in plants. The distinct experimental design requires corresponding systems operating at an appropriate field. Typical field strengths vary from 1.5 T to 3 T in clinical applications and from the earth's magnetic field at approximately 50 μT up to 23.5 T in experimental setups. In the sample the NMR-active nuclei's magnetic moments (the spins) are precessing at a field-dependent frequency, thus enabling the user to manipulate the net magnetization. A radio frequency (RF) coil sends an excitation pulse into the sample and receives the resulting signal emitted from the sample. In order to localize the signal in three dimensional (3D) space, so called magnetic *gradient* fields are used for imaging. The strength of the field at each point along the gradient corresponds to its location, and thus encodes the spatial information into the measured signal. The facility to vary the strength of the magnetic field gradients allows the signal to be transformed into a multi-dimensional image. The pulse sequence (also called the NMR sequence) and the coil both have to be optimized to the sample. Various coil designs - from single loops to multi-resonant RF coils - have been proposed [23].

4.1. From equation to image

While lipid molecules largely consist of the reduced carbons, which bond a number of hydrogen atoms (rather than nitrogen, oxygen and etc.) they can be easily detected. by both, proton and carbon NMR (^{1}H NMR; ^{13}C NMR) (Fig. 1A). The resonance frequency ω of a proton's spin in a magnetic field is directly proportional to the product of the nuclear gyromagnetic ratio γ and the external field strength B_0:

$$\omega = \gamma \cdot B_0 \tag{1}$$

The molecular environment of the spins involved have a minor, yet measurable influence on the resonance frequency, referred to as the chemical shift. The diamagnetic property of the neighboring atoms' electrons exerts a shielding effect on the spin, shifting the resonance frequency

$$\omega = \gamma \cdot B_0(1 - \sigma) \tag{2}$$

The chemical shift σ is measured in ppm, its absolute value being dependent on the field strength B_0. Protons within water experience a greater chemical shift (4.7 ppm) than those with a lipid molecule (1.2 ppm for $(CH_2)_n$). The usual choice of reference molecule in ^{1}H NMR spectroscopy is tetramethylsilane. The higher B_0, the greater is the absolute chemical shift observed between water and lipid, a property which allows for these two compounds to be distinguished during measurements. In NMR spectroscopy, various molecular components of spin bearing nuclei can be recognized on the basis of σ. In the spectrum of an olive fruit acquired with a single ^{1}H NMR sequence, many different peaks can be recognized which correspond to the protons' signals according to their individual chemical surrounding (Fig. 1B). A more detailed analysis of the ^{1}H NMR spectrum of olive oil can be found in Vigli et al. [24].

The sum of a sample's spins yields a net magnetization value M_0, which is responsible for the NMR signal. Within a given NMR sequence, RF pulses influence the orientation of the magnetization. The spatial encoding of the output signal is carried out by the use of magnetic field gradients that create a frequency or phase difference between the spins. When the signal is acquired, all the information needed for spatial and spectral encoding is encapsulated by the magnetization's behavior. The physical NMR parameters proton density (ρ), longitudinal (T_1) and transversal (T_2) relaxation time are intrinsic to the subject material, and determine the amplitude of the signal in each voxel or spectrum.

The general behavior of an NMR signal S in a standard spin-echo sequence is proportional to the magnetization M_0 and can be described by

$$S \propto M_0 \cdot \left(1 - e^{-\frac{TR}{T_1}}\right) e^{-\frac{TE}{T_2}} \tag{3}$$

The Repetition Time (TR) refers to the time interval between two consecutive excitation pulses and is used to adjust the T_1 contrast, while the Echo Time (TE) determines the T_2 contrast. T_1 is the longitudinal and T_2 is the transversal relaxation time of the magnetization.

Gradients are seldom used in global NMR spectroscopy protocols. The global spectrum of a sample can be generated in the order of milliseconds. Rapid measurements are particularly advantageous where the target is to resolve the chemical composition of a sample. Global spectroscopy is not ideal for localized analysis, because it does not provide any spatial information. Spatial encoding in MRI is based on the field dependency of the Larmor frequency. The application of gradients and pulses with variable bandwidth enables slice excitation and refocusing. Phase gradients built in before data acquisition encode the NMR signal within the image slice in a given direction. During data acquisition, the next dimension is encoded by a read gradient that leads to spatially

dependent frequencies of the individual spins (Equation (1)). This frequency information is carried by the signal emitted by the sample and can be allocated during the further procedure by use of a fast Fourier transformation.

4.2. Global spectroscopy

Global spectroscopy is the optimal choice where the aim is to rapidly derive the chemical constitution of the sample. A simple example for the application of global spectroscopy on an intact maize seed is displayed in Fig. 2. Dependent on the settings of the excitation pulse, the global spectrum shows both, water and lipid (Fig. 2A), or water only (Fig. 2B) and lipid only (Fig. 2C). This information is basis for the selection of appropriate NMR protocols for further measurements. Depending on the experimental design, several nuclei can be used for NMR spectroscopy: recent applications have featured ^{1}H NMR [24,25], ^{13}C NMR and ^{31}P NMR [26].

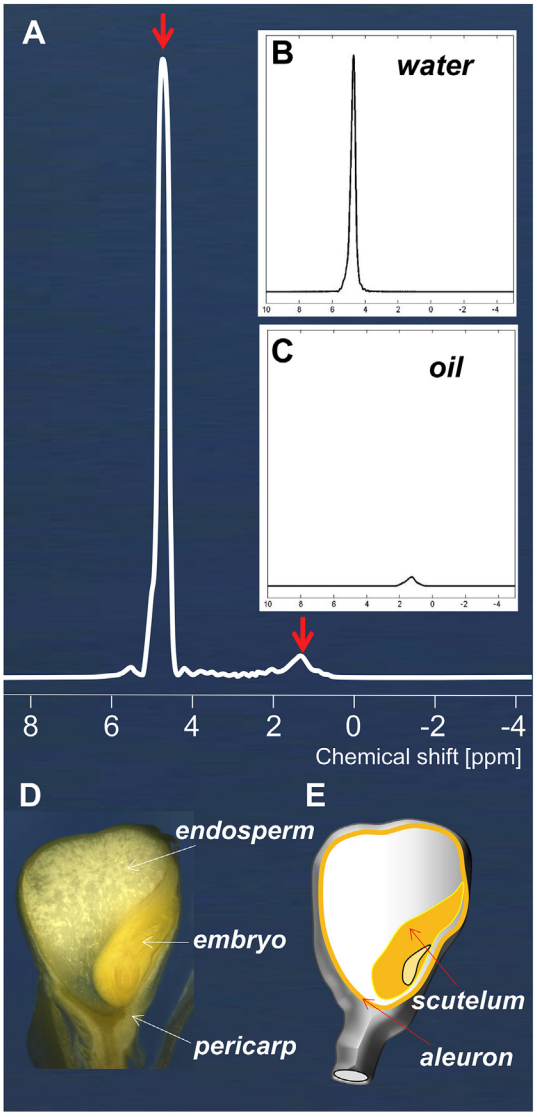


Fig. 2. Global NMR spectroscopy of an intact maize grain at a field strength of 11.4 T. (**A**) Both water and lipid are visible in the spectrum, the bandwidth of the excitation pulse was set to 8 kHz; (**B**) Only water protons are contributing to the signal, when the bandwidth of the pulse is reduced to 500 Hz; (**C**) Only lipid signal is seen, when the basic frequency of the NMR scanner is shifted by 2750 Hz additionally. (D) Cross-section of maize corn shows its internal structure; the largest organs are arrowed. (E) Lipid accumulation tissues of embryo and endosperm are arrowed.

The simplest procedure for obtaining the spectrum is referred to as the *continuous wave method*. A system for assessing the quality of oilseeds has been proposed by Colnago [27], based on a continuous wave free precession ^{1}H NMR sequence; this is capable of monitoring hundreds of large and heterogeneous seeds carrying a range of lipid content, from soybean *Glycine max* (22% lipid) through physic nut *Jatropha curcas* (35%) and peanut *Arachis hypogaea* (45%) to the macadamia nut *Macadamia integrifolia* (65%). Using a short measurement time of ca. 150 ms allows for the non-invasive quantification of lipids of up to 24,000 seeds per hour. The size of the NMR coil (in this case 18 mm) was the only limiting factor for analysis of differently sized seeds. The method does not require, in principle, either a high field or large bore magnets, and could be readily adapted to measure classes of compounds other than lipids in either seeds/fruits or food/feed.

Pulsed field gradient (PFG) techniques are widely used as an alternative to phase cycling [28]. A pulsed field gradient is a short timed pulse of spatially-variable field intensity. In this respect, the technique is key to MRI. Using a PFG sequence, Gromova et al. [29] were able to estimate the diameter of oil bodies harbored by a range of seeds by assessing the extent of diffusion of lipids out of the oil bodies. These measurements agreed well with conclusions based on optical methods. A further application of pulsed ^{1}H NMR spectroscopy is represented by the Annarao et al. [30] study of lipid accumulation and composition in the *J. curcas* seed. Here, seeds harvested at various developmental stages were subjected to lipid profiling, resulting in the detection of various free fatty acids, fatty acid methyl esters and triglycerol esters, along with small quantity of sterols. The analysis was able to define the initial stage of lipid accumulation and to estimate the proportion of the different compounds present. The experiments provided useful information regarding *in planta* lipid storage, especially in the context of up-scaling triglyceride production for the bio-diesel market.

A number of strategies based on ^{13}C labeling have been developed to explore metabolic flux during lipid accumulation, and ^{13}C NMR provides a strong platform for this purpose. Specifically, it can track the incorporation of labels into metabolites in real time. In the seed, the platform exploits the presence of a large pool of metabolites – principally sucrose and/or lipids – to provide a direct and quantitative measurement of the movement of label within the central metabolism. A recent review of the area by Allen et al. [4] has emphasized the promise of NMR as a tool for the functional analysis of plant metabolic networks, and an example of its creative use is to be found in a characterization of the central metabolism of the linseed (*Linum usitatissimum*) embryo [31]. Intact mature seeds are highly heterogeneous solid structures, so their NMR spectrum tends to comprise a number of broad, overlapping spectral lines, thereby obscuring the detailed chemical information. In MAS NMR, magnetic anisotropies within the sample are dealt with by rotating it around an axis tilted at 54.74° to the vertical. The resulting line broadening is thereby reduced, producing an improvement in resolution. Typical spinning frequencies lie in the range a few hundred to 70,000 Hz [32]. The magic angle spinning (MAS) NMR technique is a frequent choice when the sample is a solid material.

Terskikh et al. [33] demonstrated the potential of ^{13}C NMR in an *in vivo* metabolite profiling experiment carried out on conifer seeds. The profiling provided not just an accurate quantification of the seeds' lipid content, but also derived the fatty acid composition of the major storage lipids present. Free amino acids (arginine and asparagine) were detectable during both germination and early seedling growth. ^{1}H and ^{13}C NMR have been successfully used to predict seed viability and to detect the presence of developmental aberrations in conifer seed. While the quality of the ^{1}H spectra

obtained from intact seeds was inadequate, the application of a ^{13}C MAS NMR protocol was able to monitor compositional changes in the seeds during aging. Specifically, these changes were thought to reflect the loss of polyunsaturated fatty acids following the oxidation of storage lipids [34]. A correlation could finally be established between the strength of the signal associated with unsaturated ^{13}C atoms and viability. Sarkar et al. [35] combined MAS NMR with liquid state ^{1}H NMR to reveal the mechanisms underlying the water uptake of germination sesame (*Sesamum indicum)* seed. A positive correlation was observed between water uptake and lipid consumption during germination.

The application of ^{1}H and ^{13}C MAS NMR to *Arabidopsis thaliana* seedlings provided Wheeler et al. [36] with the means to contrast wild type plants with a cellulose-deficient mutant. Diffusion-weighted spectra were used to identify signal from molecules with restricted diffusion, such as lipids. Acquiring two dimensional HSQC (heteronuclear single quantum coherence) spectra was informative regarding the molecular connectivity between ^{1}H and ^{13}C, and provided a metabolite fingerprint. The combination of these methods enabled the authors to produce a comparison of the fatty acid composition of the wild type vs mutant seeds, and also to reveal differences in the way in which stored energy was processed. Through the use of other NMR methods (e.g. 1D ^{13}C Cross Polarization and 1D solid-state ^{13}C NMR spectra, both for detection of the rigid compounds such as starch, cellulose, and lignin) differences were also identified in the content of lignin, methanol, glutamine, phenylalanine, starch and nucleic acid.

4.3. Localized spectroscopy

Plant samples are usually consisting of distinct organs/tissues, as exemplified (in Fig. 2D). As a plant seed consists of three major organs (seed coat, endosperm and embryo), each has its specific set of functions, and each also influences the seed's size, composition and metabolism/growth. Observing the behavior of these discrete structures *in vivo* (e.g. distinct pattern of lipid accumulation in embryo and endosperm: Fig. 2E) during the course of the seed's development, germination and response to environmental cues is of both fundamental and applied interest. Unfortunately, described above global spectroscopy cannot supply the necessary data; instead, to obtain a localized spectrum, the selective excitation of a defined portion of the sample is needed (Fig. 3A).

Point-resolved spectroscopy (PRESS) requires one slice excitation pulse and two orthogonal slice refocusing pulses in order to acquire a voxel-specific spectrum (Fig. 3B, C). Srimany et al. [37] were able to track the lipid content in embryo of the areca palm fruit (*Areca catechu*) using a PRESS sequence, and demonstrated its increase over time. At the same time, data regarding the water, sugar and alkaloid content of the seed were collected.

4.4. Spatially resolved imaging based on chemical shift

Spatially resolved spectra can also be acquired by exploiting chemical shift imaging (CSI). Using only phase encoding gradients, a spectrum is acquired for each voxel in the imaged volume, which allows in the following the selection of regions of interest in either the image or the spectral dimension, either the associated spectra or the images to be displayed. Compared to what is produced by standard NMR spectroscopy, the information gained by CSI is expanded over two additional spatial dimensions (Fig. 4A). CSI is a phase-encoding only method, which requires a relatively long TR in order to avoid signal saturation. This results in a prolonged measurement time, so most experiments are restricted to two dimensions. Nevertheless, the spectral information can be used to image a variety of chemical compounds (Fig. 4C, D).

Glidewell et al. [38], using CSI to measure the development of the barley grain, were able to generate separate images for water, lipids and sugars. NMR spectra captured at a series of developmental stages and focused on specific regions of the caryopsis showed, for example, how lipid content increases in the embryo over time, while the content of soluble carbohydrate declines in the endosperm.

5. Chemical shift selective imaging (CSSI)

If detailed chemical information about the NMR spectrum is not so important, then Chemical Shift Selective Imaging (CSSI) is the method of choice for acquiring high resolution images from compounds of interest and within a moderate measurement time. The CSSI method takes advantage of the dependence of a proton's resonance frequency on its molecular context. 3D datasets can be acquired, so that the whole of a seed or a large portion of a plant can be imaged (Fig. 4E). The measurement time of a three dimensional CSSI dataset is in the range of a standard 2D CSI dataset with a similar in-plane resolution, since the CSSI-method utilizes a read gradient during data acquisition that accelerates the data acquisition in a spatial dimension. Three alternative experimental approaches have been developed and are described below:

5.1. No suppression, no selection approaches

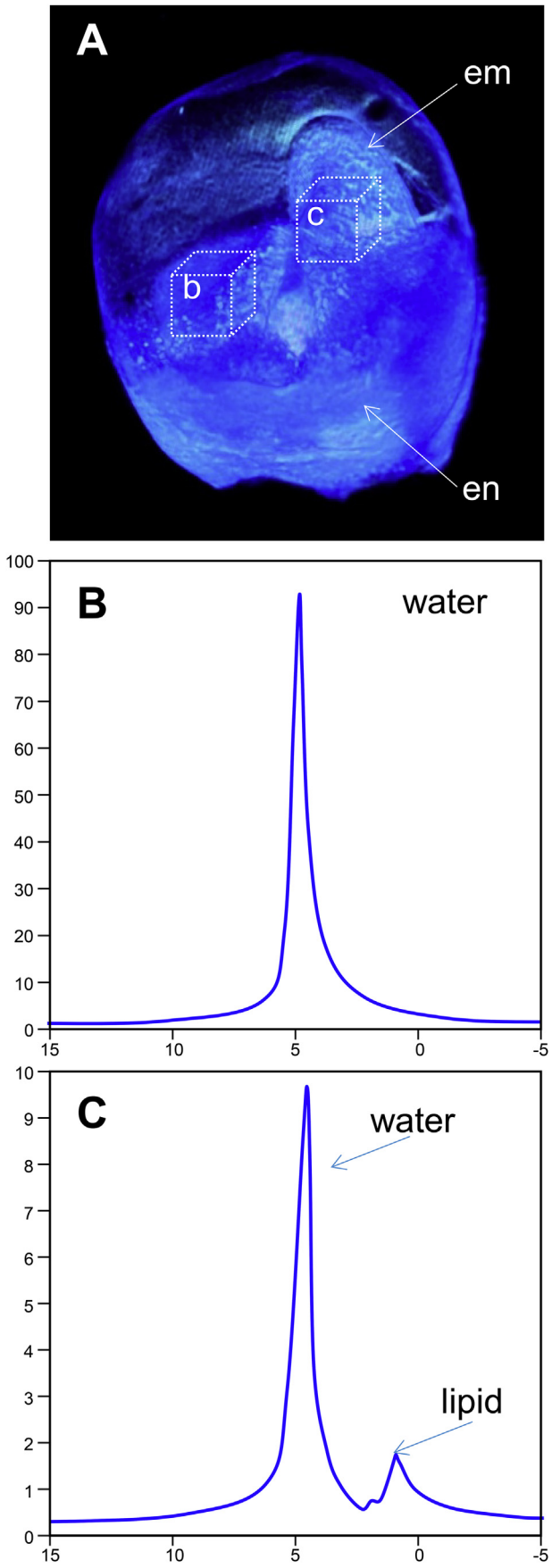
When the object of the NMR analysis is a sample in which lipids are the only NMR active component (for example dry mature seeds), the sequence can be run without any major preparation. Bound water does not contribute to the resulting image because of its short (in the order of 1 ms and below) relaxation time. With this method NMR imaging has reached the highest in-plane resolution in lipid imaging in plants [83].

Fuchs et al. [39] derived virtual models of the submillimeter tobacco seed based on measurements made from >200 seeds from both a wild type cultivar and a transgenic line (engineered to express in the seed a gene encoding a plastidial phosphoenolpyruvate/phosphate translocator); to achieve this, the seeds were assayed in bulk using a standard spin-echo sequence, which was possible as no water signal was detectable in dry seeds. The seeds were suspended in oil for the purpose of correcting the coil's RF-field and calibration. A comparison of the lipid models of the wild type and transgenic seed revealed clear differences in the pattern of lipid accumulation in the seed organs (endosperm and the embryo). A correlation between seed size and lipid content was detected in the transgenic, but not in the wild type line.

Terskikh et al. [34], using the similar assay to investigate seed viability of the western red cedar (*Thuja plicata*). Measurements indicated that non-viability was associated with a markedly reduced “liquid” lipid content, a conclusion which was not drawable from X-ray images [34]. The suggestion was that germination capacity is dependent on “liquid” lipid content, since the oxidation of this fraction, which occurs when the seed coat is damaged, tends to promote lipid polymerization. Investigation of the influence of aging on lipid distribution in the barley and wheat grain was done by applying a standard spin-echo sequence; no suppression of water or chemical shift selection was needed because the moisture content of the grain was low (Table 1). Aging had no perceptible effect on either the spatial distribution or the quantity of lipid present.

5.2. Solvent suppression approaches

Solvent suppression is generally exploited when more than one target compound is NMR active. Water suppression is particularly



useful for the detection of sugars and protons attached to lipid molecules. The sequence can be adjusted so that detection is focused on, for example, only water or only lipids. This approach facilitates the acquisition of a chemical shift selective dataset more rapidly than is possible using CSI, but only one component at a time can be tracked.

Hong et al. [40] in an exploration of soybean and groundnut seeds, emphasized the necessity for reliably suppressing specific compounds (solvent). In the absence of solvent suppression, image quality can be severely deteriorated due to phase cancellation and "shadow" artifacts. The artifacts are created by the sum of the two complex signals (in the overlap of the water and lipid signals), while inclusion of solvent suppression allowed the desired signals to be clearly distinguished. Fuchs et al. [39] described a solvent suppression scheme able to produce both water- and lipid-selective images of a tobacco capsule. These NMR images produced both an improved level of resolution over light microscopy-derived ones, while also providing a semi-quantitative measure of the capsule's and seeds lipid content (Table 1).

5.3. Frequency selective approaches

NMR imaging in an ultra-high magnetic field allows the user to include frequency-selective excitation and refocusing pulses. The chemical shift of protons is measured in parts per million (ppm) in relation to the NMR system's basic proton frequency. This shift is equivalent to an absolute frequency difference of about 3 kHz for lipid protons compared to water, when the field strength is 20 T (yielding a ^{1}H-frequency of approximately 850 MHz). The spectral peak of a compound has a finite bandwidth, typically in the range of 100 (wet sample) to 2000 Hz (dry sample). The frequency selective approach requires an accurate shim, thus an equalization of the basic Larmor frequency within the sample. The pulse bandwidth needs to be adjusted according to the NMR frequencies of the compounds to be imaged, after which, depending on the imaging software available, interleaved measurements based on a series of pulses can be performed. A narrow pulse bandwidth, which is required for a compound-selective excitation, lengthens the duration of the NMR pulse and thus increases the minimal echo time. In most applications this small elongation of the minimal echo time is in the range of 1–2 ms, therefore it does not impose a significant restriction for lipid imaging. The influence of a longer echo time on the SNR in the resulting image is therefore negligible. After the shim of the magnetic field and the bandwidth of the pulses are optimized, no solvent suppression is required.

The ripening process of olive fruit has been studied by imaging the distribution of water and lipids using frequency-selective pulses [41]. The experiments revealed the movement of water from the inner to the outer mesocarp, while lipids accumulated in the pulp. Cultivars could be distinguished on the basis of these NMR parameters. The development of the barley grain has been characterized through independently acquired water and lipid datasets, using CSSI [42]. The resulting high resolution images were used to clarify the internal anatomy of the grain and the mechanics of lipid storage. Furthermore, the NMR parameters TR and TE were chosen appropriately for minimization of influences by relaxation processes within the sample. A calibration procedure involving the gas

Fig. 3. Spatially resolved ^{1}H NMR spectroscopy on an intact maize grain. (**A**) The 3D ^{1}H NMR image displays internal structure of corn in 3D (in blue): cubes *b* and *c* (in white) represent voxel, which were analyzed; (**B**) The spectrum acquired in the region of endosperm; only water signal is apparent; (**C**) The spectrum acquired in the region of embryo; both water and lipid signal are apparent. Comparison of localized spectra (B) vs (C) reveals difference in composition of embryo *vs* endosperm (note distinct scales!). Abbreviation: em-embryo; en-endosperm. See for details Fig. 2.

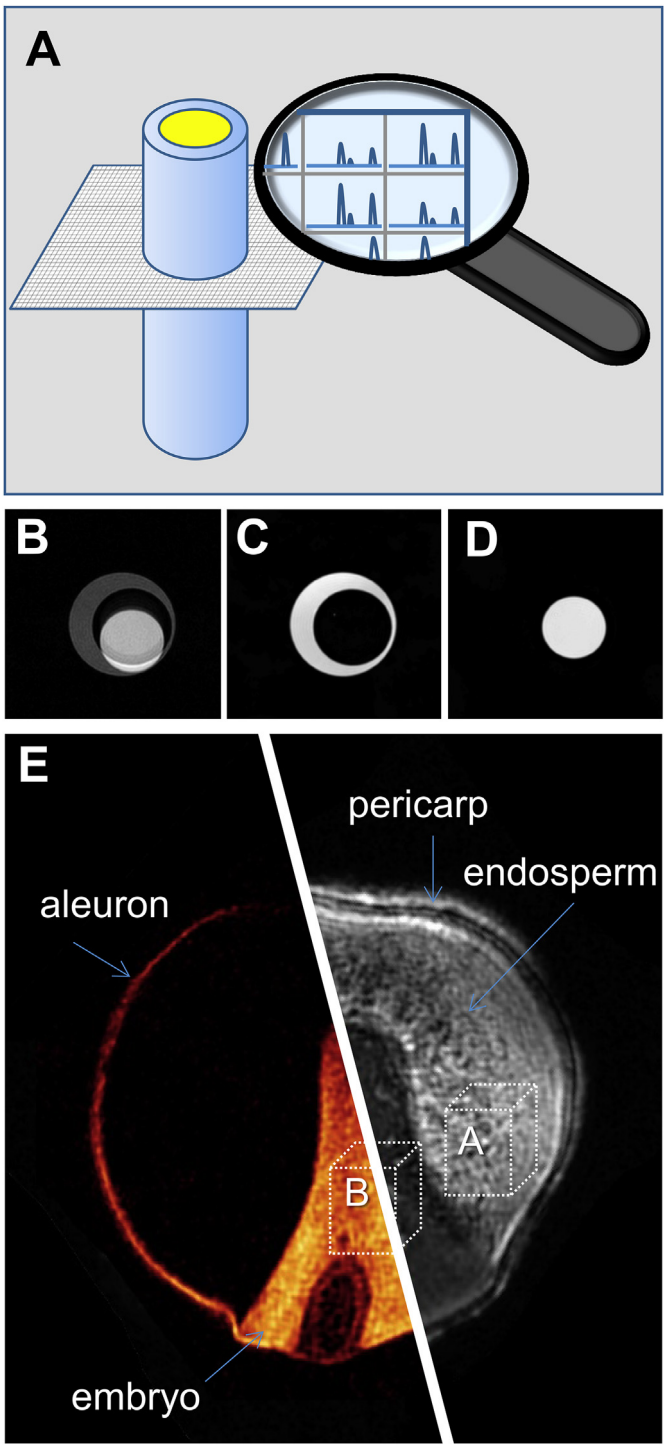


Fig. 4. Chemical Shift Imaging of water and oil containing samples. (**A**) Scheme shows an oil-filled tube (yellow) inserted into a water-filled tube (blue). CSI generated NMR spectra at any point of phantom (magnifying lens). Dataset is used for reconstruction of the whole image; (**B**) Image of the phantom (cross-section) by standard imaging techniques: oil and water can be seen. Image suffers from the displacement of „oil *vs* water" which is an example of the chemical shift artefact. (**C, D**) Images of the same phantom by CSI-application show accurate separation of water (C) and lipid (D) avoiding CS-artifacts. (**E**) CSS-image of intact maize corn shows oil (left) and water (right) distribution within the same section. Embryo and aleurone show high lipid signal, whereas endosperm and pericarp show high water signal. See Fig. 2 and 3 for more details.

chromatography of selected dissected areas of a target seed could be achieved. Based on this information, the whole sample could be quantified regarding the absolute lipid content based on the 3D NMR dataset. The integrated data were then used to assess lipid concentrations (in the micromolar range) within the grain (Table 1). When the same approach was applied to the soybean seed, there was a high level of agreement with the outcome of destructive gas chromatography [42]. Hayden et al. [43] used a CSSI sequence to identify the endosperm of the oat grain as being the predominant site for lipid accumulation. Spatially resolved NMR was able to distinguish between two closely related oat cultivars which differed in their grain lipid deposition and content. Horn et al. [44,45] applied mass spectrometric imaging (MSI) and compared it with three dimensional CSS imaging of lipids in cotton seeds (*Gossipium* sp.) as well as in false flax seeds (*Camelina sativa*) and identified some putative differences between genotypes with respect to metabolic processes underlying lipid production within the embryo.

One application of high resolution MRI takes advantage of a water-suppression scheme prior to the lipid imaging sequence. This enabled visualization of steep gradients in oil deposition with high resolution and within the living plant tissues. For example, the lipid distribution in the tiny embryo of oilseed rape (*Brassica napus*) was visualized under *in vivo* and *in vitro* growth conditions [46]. The authors define the spatial/temporal relations of lipid topology to those established for the accumulation of starch and storage proteins, cellular growth, photosynthetic activity, and metabolite pattern. This study demonstrates how the embryo is able to make local metabolic adjustments to its environmental and growing conditions and provide a mechanistic view on the role of seed architecture for embryo metabolism in living seed. A frequency-selective spin-echo sequence was used by Rolletschek et al. [47] to characterize the three dimensional distribution of lipids in the oat grain (number of wild type plants and cultivars). The data was used to estimate the volume and lipid content of the distinct seed organs. Kovalchuk et al. [48] adapt the MRI-method for comparison between the lipid fraction in both the embryo and endosperm of a single bread wheat grain (*Triticum aestivum*), which showed that the embryo and aleurone layer together represent the major storage site for grain lipids. In a contrast between a wild type and a transgenic wheat authors noted differences in the distribution of lipids, as well as in grain organ size; these alterations were associated with metabolic perturbation induced by the presence of the transgene.

An elegant CSSI based approach is an interleaved measurement of two or more components. This procedure saves measurement time by applying component-selective pulses in distinct time sequence, which is designed so, that one component is excited during the interval reserved for the relaxation of the other components. High resolution NMR imaging proved capable of making interleaved selective measurements (water and lipid). The analysis of seed imbibition and germination represents a major application of frequency-selective pulses in combination with interleaved volume acquisition. Terskikh et al. [49] imaged imbibed seeds of pine (*Pinus monticola)* at hourly intervals, studying both water uptake and changes in the lipid content both inside and at the surface of the megagametophyte. Similar success was reported by Fuchs et al. [39] during the imaging of the tobacco capsule. The water dataset delivered information regarding the hydration of the capsule, and identified the vascular bundles, along with the placental stalk and bundles, as being the most strongly hydrated portions of the capsule. In addition, it made possible a non-destructive estimate of the number of seeds harbored by the capsule. Hundreds of seeds were simultaneously imaged at high resolution, sufficient to assess lipid gradients inside of individual

Table 1
Remarkable facts about MRI of plant lipids.

Achievement	NMR method	Magnetic field strength	Author (year)
Lowest magnetic field used for lipid-imaging	2D Spin-Echo No suppression No selection	1.5 T	Kotyk et al. [50]
Highest magnetic field used for lipid-imaging	3D Spin-Echo	20.0 T	Borisjuk et al. [81]
Smallest seed ever imaged (tobacco, *Nicotiana tobaccum*)	3D Spin-Echo No suppression No selection	17.6 T	Fuchs et al. [39]
Largest seed used for lipid-imaging (coconut, *Cocos nucifera*)	2D Spin-Echo No suppression No selection	4.7 T	Jagannathan et al. [82]
Highest in-plane resolution ever reached (6 μm × 6 μm)	3D Spin-Echo No suppression No selection	17.6 T	Schneider et al. [83]
Highest number of **simultaneously** measured seeds (2592)	Multi-Slice Spin-Echo No suppression No selection	1.5 T	Kotyk et al. [50]
Highest sample throughput at 25 μm isotropic resolution (2.6 min/seed)	3D Spin-Echo No suppression No selection	17.6 T	Fuchs et al. [39]
First **quantification** *in vivo* with tissue specific resolution (31 μm × 31 μm)	2D Spin-Echo Frequency-selective pulses	11.7 T	Neuberger et al. [42]
First lipid visualization in **germinating seed**	2D Spin-Echo Frequency-selective pulses	9.4 T	Foucat et al. [84]
First imaging of plant oilseed **digestion by human** (sunflower, *heliantus annuus*)	Gradient-Echo Frequency-selective pulses	3.0 T	Hoad et al. [75]
Imaging of distinct **pharmaceutical compound** of lipid fraction in intact fruit (thymol; *Carum copticum*)	2D-Spin-Echo Frequency-selective pulses	7.1 T	Gersbach et al. [85]
First application as a tool for non-invasive **taxonomy** (herbarium material)	CSI	7.1 T	Glidewell et al. [86]
Lipid imaging as a tool for **contamination** detection	2D Spin-Echo Frequency-selective pulses	7.1 T	Tan at al. [87]

submillimetre seeds (Table 1).

The NMR-based assay of seed lipid content, composition and topology (e.g. using CSSI and interleaved selective measurements) has a number of potential applications, in particular providing a means to test and optimize transgenic strategies aimed at the manipulation of seed size, seed number, and lipid content in fruits of various species. A few examples of Chemical Shift Selective images of lipid in seeds of crops are exemplified in Fig. 5 and summarized in Table 1.

6. Applications of low field NMR

The advantages of low-field NMR over high-field MRI lie in the simplicity of sample preparation, the rapidity of the assay and its amenability to high throughput analysis. The growing interest in low-field MRI reflects the possibility of constructing portable devices able to perform *in vivo* measurements outside a laboratory. The low field strength reduces the frequency difference between water and lipid, thus, the separation between the water and lipid signals either requires high grade shimming of the sample or an application of a frequency-independent approach. Low-field NMR (as compared to the high field) is less appropriate for accurate/high resolution lipid imaging in living plant tissues with high water content.

Advantage can be taken of the component-characteristic relaxation parameters T_1 and T_2. Benchtop systems based on the Time-Domain (TD) NMR method have been designed to deliver a rapid analysis of the sample's chemical content. A strong weighting of T_1 and T_2 is used to obtain a single measurement, which in reference to a calibration database, allows conclusions to be drawn regarding the content of lipid, water, carbohydrates and etc.

The simultaneous measurement of 2592 corn kernels was performed by Kotyk et al. [50]. The sequence parameters were chosen in such a way that only the lipid was contributing to the signal. Quantitative results were determined by using the measured signal and the known weight of each seed combined with the oil reference measurement. Single corn seeds were also analyzed with a 0.5 T spectrometer using TD-NMR, the results of both approaches showed a good agreement with invasively acquired lipid data. Gao et al. [51] attempted to quantify the lipid content of the algal species *Chlorella protothecoidese* using TD-NMR, and found that predictions were more consistent with analytically obtained values than with those generated by histological staining; furthermore, the low coefficient of variation associated with the TD-NMR data suggested the method to be highly reproducible. Sharaani et al. [52] applied a field strength of 2.35 T instrument to profile water and lipids in the oil palm fruit (*Elaeis guneensis*). From the resulting multi-echo dataset, a multi-exponential decay function was fitted to derive separate maps for both T_2 and M_0 values. The NMR output obtained was used to distinguish three different T_2 components, associated with the water content, the lipid content and the shell. When the absolute T_2 values, along with the associated magnetizations (M_0), were monitored during fruit ripening, a distinct increase in lipid content over time was apparent.

The temperature dependency of T_2 was explored by Carosio et al. [53] using spin-echo and continuous wave free precession sequences. The temperature of groundnut, soybean and macadamia seed was estimated from their lipid T_2 value, a parameter which is dependent on the viscosity of the lipids. This method enabled them to calculate the thermal diffusivity of the seeds by placing them in a low temperature bath and monitoring the course of the temperature. The NMR approach provided information of the seed's internal physical properties that were not accessible with standard methods, but could be essential by bioengineering of oil seed crops [16].

Iulianelli et al. [54] measured seed ^1H T_2 values in a collection of cassava (*Manihot esculenta*) accessions. Substantial variation in lipid content was observed between different genotypes of seeds

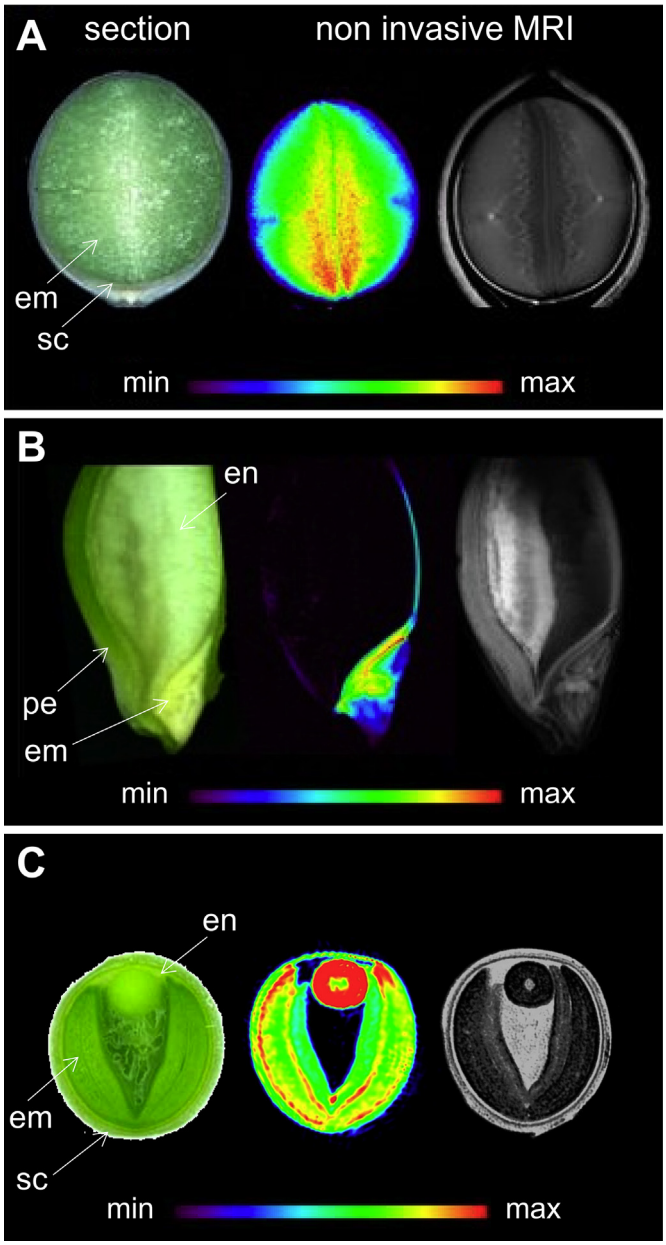


Fig. 5. Chemical-Shift-selective images (CSSI) of oil bearing seeds. The morphology of cross-section (left column) and the corresponding NMR images of lipid (middle column) and water (right column). (**A**) The soybean seed exhibits a lipid gradient towards the center of the seed. See for details: *Borisjuk* et al. [61]. (**B**) The lipid in the barley is accumulated in embryo and distinct regions of endosperm (aleurone). See for details: *Neuberger* et al. [42]. (C) In the oilseed rapeseed, the lipid is accumulated in the radicle and cotyledons of embryo, as well as in the endosperm. See for details: Borisjuk et al. [46].

and in addition, the authors were able to derive estimates of polysaccharide and fiber content in seeds. Rolletschek et al. [47] used a benchtop TD-NMR system to characterize a collection of some 3000 oat accessions for grain weight, along with lipid, water and carbohydrate content. The estimated throughput of up to 1400 samples per day proved the capability of low field NMR to acquire quantitative information in a non-invasive manner and with the high throughput.

To the best of our knowledge, there has been no imaging of lipid in plant seeds performed using low field benchtop NMR instruments.

7. Some practical considerations for lipids imaging *in planta*

High magnetic field strengths are generally favored for *in vivo* NMR imaging, but the biological effects of these fields cannot be discounted [55,56]. Plant specimens appear to be less sensitive to magnetic fields than are animal ones [56,57], so both long duration exposure and the use of short, intensive excitation pulses can be attempted [58].

However, most plants are highly sensitive to the *direction of gravity*, so the orientation of the bore of NMR system needs to be vertical, especially during long duration experiments. Also, living plant needs to be supported by supplying water, nutrients and light, requirements which have driven the design of customized controlled climate chambers. The inclusion of a climate control device within the NMR chamber is restricted by space limitations, so attempting imaging of some whole plants can be hindered by the insufficient bore diameter of the instrument. For example, the high field NMR systems equipped with an imaging gradient capacity have a much smaller vertical bore then any horizontal NMR system which are designed primarily to image a prone human subject. In such situations, hardware design becomes an important issue, and needs to be factored into the experimental design [46,59,60].

Chemical shift protocols do provide the experienced user with a deal of experimental flexibility, but should be used with precaution. Where the presence of more than one NMR visible component is ignored, chemical shift artifacts can compromise image quality. In read-encoding-direction the water and the lipid images overlap, the lipid signal is slightly shifted in the read direction (Fig. 4B), as exemplified by Hong et al. [40], Borisjuk et al. [61], Mazhar et al. [62].

As an example for a thorough NMR experiment one could start with (1) the detection of the basic frequency which reveals the global spectrum of the sample. Here the user has to choose the experimental parameters/procedure. (2) Fast detection of the longitudinal relaxation parameter T_1 enables for correct choice of the sequence parameters. (3) CSSI-sequence with a water suppression module can be used [63]. Finally, (4) the acquisition of multiple spin-echoes allowed for T_2-calculation after the experiment in order for further quantification procedures based on Neuberger et al. [42].

8. Plant-specific aspects of NMR technology

NMR imaging was initially developed as, and still remains, a technology used for clinical diagnostics, although it is gradually finding uses in food science [13]. Plant biology represents a budding field for both MRI analysis and associated computer visualization [63,64], and often adopt technological innovations from other areas. While the means of acquiring lipid profiles is similar for both plant and non-plant subjects, most protocols developed for animal/medical subjects cannot be directly applied to a plant sample.

A major difference between plant and animal cells is the cellulosic cell wall, but in addition, many plant tissues feature a significant intracellular space filled with either air or fluid. The presence of trapped air induces heterogeneity in an applied magnetic field, which can give rise to artifacts during NMR [65,66]. Tissues which have a high water content, and particularly the presence of droplets of liquid, produce high intensity NMR images, due to the relatively high T_2 value associated with liquid compounds. The imaging of tissues with a low water and/or low lipid content is hindered by a too rapid decay in signal, as a result of high spin-spin interactions and a fast longitudinal relaxation time. Such materials require short echo time sequences and some averaging of the signal may be necessary. For example, Spiral Imaging, is a technique efficiently used for lipid analysis in medical diagnostics [67], but its

application to plants could be challenging as it requires a highly homogeneous magnetic field, along with long readout times in order to achieve a high spectral resolution. The Dixon Imaging protocols, widely used to distinguish fat from water, is rather prone to artifacts when applied to plant samples, largely because of plant tissue heterogeneity. Even Multipoint Dixon Imaging methods, developed to deal with heterogeneity, cannot be readily transferred to plant subjects because of the low signal to noise ratio (SNR) and the overlong measurement times required [68].

To conclude, NMR has been able to reach an image resolution close to cellular size *in vivo* (Table 1) when the combination of NMR hardware, NMR protocol and the object of interest are well-considered (see for review [61]).

9. Future perspectives

The importance of studying lipid metabolism *in vivo* is stimulating the application of NMR to monitor metabolite flux and diffusion, as well as to quantify cell size, tissue water content and metabolic activity. Improvements may come from combining several NMR-based techniques, which could increase the information content of the imaging, as well as from technical developments in NMR technology.

The tailoring of NMR to plant subjects does not generally require any hardware remodeling, but rather relies on the creative design of measurement programs and pulse sequences. For example, ^{1}H NMR structural imaging could be combined with ^{13}C NMR imaging [34], and/or MAS NMR spectroscopy. So far, both MAS and MRI applications have been largely confined to biomedical situations, but there are already some plant-based examples, such as the metabolic profiling of *J. curcas* infected with mosaic begomovirus [69].

Current NMR technology is able to derive lipid topology, describe the metabolic environment and deduce the structure of lipid-bearing tissues. Its weakness lies in a poor level of chemical sensitivity/resolution compared with conventional technologies such as mass spectrometry and optical microscopy. Consequently, there is considerable interest in integrating NMR-based approaches with technologies which can compensate for limited spatial resolution (micro-computerized tomography and X-ray imaging, see Ref. [70]), sensitivity (positron emission tomography labeling experiments, see Ref. [4]) or chemical resolution (MALDI-mass spectroscopy on cotton (*C. sativa)* seeds, see Ref. [45]). At present, NMR provides the ideal means of obtaining a broad-brush analysis of lipid topology, while other platforms (mass spectroscopy-based metabolomics etc.) are used to fill in the details.

In parallel, future applications of lipid imaging by means of NMR will focus on improvement of chemical resolution, for example differentiation of the lipid compounds visible in the NMR spectrum (localization of saturated and unsaturated fatty acid components (FACs) in oil-bearing seeds), which could provide essential information about seed composition and fitness. The imaging of dry compounds is impeded by a signal decay that is too fast for most MRI experiments. The utilization of sequences, that enable the acquisition of these substances, is a further challenge for sequence design. A promising approach could be the adaption of ultra-short echo-time (UTE) sequences, that are widely used in clinical studies [71]. First applications of this imaging technique [72] have shown an increase of information about the investigated sample.

Significant progress is expected in developing benchtop and portable NMR devices, which would greatly simplify the mass screening of oilseed germplasm, and the characterization of transgenic and mutant lines [39,47,73]. The hope is that, in due course, instrumentation will become available which is both affordable, easily maintainable and capable of high throughput use. In order to promote the sharing of data, another priority is the creation of appropriate, open-access databases.

Human health is dependent on the quality/quantity of lipid supplied with food/plant/seed. The digestion of the sunflower-seeds by a human was monitored *in vivo* using MRI [74,75]. MRI is capable to trace lipid as a biomarker and as a transport vehicle (e.g. for contrast agents and fluorescent labels [76]. Nowadays MRI assists investigations/visualization of lipid during vital processes, for example the development of human brain (e.g. myelination [77], gastrointestinal functions [78], alteration of vascular vessels, as well as therapeutic treatments (e.g. effects of lipid levering therapy), nutritional diseases and cancer [79]. MRI experiments on animals display how lipid quality of feed affects lipid composition of adipose tissue of consumers, their metabolism and fitness in general [78,80]. There is hope that harnessing MRI for non-invasive investigation starting from seed-, feed-, food-to the medical-science will narrow the gaps between these fields of science and lead to better understanding of lipid metabolism in general, to the improvement of nutrition quality and prevention of nutritional and other diseases.

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