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Microcoil MRI of plants and algae at ultra-high field : an exploration of metabolic imaging

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An exploration of metabolic imaging

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An exploration of metabolic imaging

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ABREVIATIONS

1D	One-dimensional
2D	Two-dimensional
3D	Three-dimensional
ACQ	Acquisition
ADC	Apparent Diffusion Coefficient
Asp	Aspartate
B_0	Magnetic field flux
B_1	RF pulse
BW	Bandwidth
C	Capacitance
CCT	Correlated Colour Temperature
CNR	Contrast-to-Noise Ratio
COSY	Correlation Spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill
CSI	Chemical Shift Imaging
CSSI	Chemical Shift Selective Imaging
CT	Computed Tomography
D_{coil}	Coil Diameter
DQD	Digital Quadrature
D_{voxel}	Voxel diameter
DW	Diffusion Weighting
DW-CSI	Diffusion Weighted Chemical Shift Imaging
DWI	Diffusion Weighted Imaging
Fig.	Figure
FLASH	Fast Low Angle Shot
FLIM	Fluorescence-Lifetime Imaging Microscopy
FOV	Field of View
FWHH	Full Width at Half Height
GABA	γ -Aminobutyric acid
GC	Gas Chromatography
G_D	Diffusion Gradient
GE	Gradient Echo
G_x, G_y, G_z	Imaging gradients in X, Y or Z direction
h	Planck's constant
$\hbar$	Reduced Planck's constant.
HR-MAS	High Resolution Magic Angle Spinning
ID	Inner Diameter
ISA	Image Sequence Analysis
JPRESS	J-resolved Point Resolved Spectroscopy
L	Inductance
LC	Liquid Chromatography
LCOSY	Localised Correlation Spectroscopy
MGE	Multiple Gradient Echo
MIP	Maximum Intensity Projection
MR	Magnetic Resonance
MRM	Magnetic Resonance Microscopy

MRS	Magnetic Resonance Spectroscopy
MS	Mass Spectroscopy
MSI	Mass Spectroscopy Imaging
MSME	Multi-Slice Multi-Echo
M_{xy}	Transversal magnetisation
M_z	Longitudinal magnetisation
NA	Number of Averages
NEX	Number of Excitations
NMR	Nuclear Magnetic Resonance
PBM	peribacteroid membrane
PFD	Perfluorodecalin
PRESS	Point Resolved Spectroscopy
RAREVTR	Rapid Acquisition Relaxation Enhancement with Variable TR
RF	Radio frequency
ROI	Regions of Interest
SE	Spin Echo
SNF	Symbiotic Nitrogen Fixation
SNR	Signal-to-Noise Ratio
SRF	Spatial Response Function
T	Tesla
T_1	Longitudinal relaxation time
T_2	Transversal relaxation time
TE	Time of Echo
TR	Time of Relaxation
v/v	Volume/Volume
VAPOR	Variable Pulse power and Optimized Relaxation delays
VOI	Volume of Interest
WT	Wild Type
γ	Gyromagnetic ratio
δ	Diffusion gradient duration or Chemical Shift
Δ	Diffusion gradient separation
τ	Pulse duration
ω	Resonance frequency

1 GENERAL INTRODUCTION

Society has a pressing need for sustainable food and energy production, as well as halting any further environmental degradation by human activity, to avert catastrophic changes to the global climate (IPCC 2014). Central to many of challenges facing humanity, such as food and energy security, are metabolic processes. The metabolism of an organism is dependent on and shaped by its environment. For multi-cellular organisms, the anatomy plays a role as well, and the interactions of metabolism tissue function may be collectively described as physiology. The intricacies of the metabolism take many shapes, whether it be through tissue specialisation in the form of organs, *e.g.* root nodules in *M. truncatula*, or concentration gradients of nutrients or metabolic intermediates (Nap and Bisseling, 1990; Xiao *et al.*, 2014; Pfau *et al.*, 2016). Understanding the processes within organisms may potentially aid in answering challenges facing humanity, related to energy and food security.

Metabolic profiling and metabolomics are a long-standing tradition in plant biology, due to the strong relation of metabolism with plant growth. Over the past decades, several advanced techniques have become available including gas chromatography (GC), liquid chromatography (LC), mass spectroscopy (MS) and their combinations: GC-MS, LC-MS and GC-LC (Barsch *et al.*, 2006; Sumner *et al.*, 2014). Since metabolic profiles can vary across tissues, there is a need to visualise metabolic profiles using localised techniques. Surface mapping of metabolites is possible with techniques such as mass spectroscopy imaging (MSI) and surface-enhanced Raman spectroscopy (Ye *et al.*, 2013; Boughton *et al.*, 2016; Espina Palanco, Mogensen and Kneipp, 2016). While the described techniques are capable of resolving many metabolites, sample material must frequently be pooled in order to obtain sufficient quantities of material and imaging is mostly restricted to surface tissue. In contrast, MRI is a 3D imaging technique based on detecting nuclear spin, capable of resolving tissue anatomy and metabolic profiles *in-vivo* or *in-situ*, though MRI is typically less sensitive compared to other techniques. In many applications, the previously mentioned techniques may be used in combination with NMR or MRI to complement each other.

This thesis uses a flexible approach to apply the latest development in MRI to two model systems, *B. braunii* and *M. truncatula*. These model systems were selected based on their importance for research for a sustainable society (Barker *et al.*, 1990; Metzger and Largeau, 2005; Tasić *et al.*, 2016). In order to map the variation of metabolic profiles across tissues, Magnetic Resonance Imaging (MRI) is used extensively, due to its ability to non-invasively study both anatomy and spectroscopy (Van As and van Duynhoven, 2013). MRI methodology was developed for these applications, through protocol development, coil selection and home-built microcoils for ultra-high field strengths.

1.1 THEORY OF MAGNETIC RESONANCE IMAGING

Nuclear Magnetic Resonance is made possible by a property of nuclei called spin. NMR was first observed experimentally in independent studies by Purcell and Bloch in 1946

(Bloch, 1946; Purcell, Torrey and Pound, 1946). NMR instruments are capable of revealing detailed information on the nuclear spins contained in a sample. These spins are manipulated using a combination of strong magnetic fields and radiofrequency pulses. The origin of spin can be described accurately by quantum mechanics (Levitt, 2001). Only nuclei with an uneven number of protons or neutrons exhibit spin. For instance, hydrogen (^1H) contains only one proton and has spin $S = \frac{1}{2}$, and its isotope deuterium (^2H) has one proton and one neutron and has spin $S = 1$. Spin is interesting as a physical property because it has no clear parable in daily life, *e.g.*, like an apple falling from a tree, such as in the case of gravity. However two useful analogies may be found to aid in understanding spin, the first being a bar magnet, and the second is that of a children's spinner toy (rotational motion).

The first analogy, a bar magnet, helps explain the reaction of nuclear spin to an external magnetic field (B), measured in Tesla (T). In the absence of a magnetic field, the orientation of the spin of nuclei is randomly distributed. In other words, the energy levels are degenerate, see Fig. 1.1. However, when an external magnetic field is applied, the energy levels of the quantified spins split.

The number of possible spin states depends on the type of nucleus. Here we consider only the spin- $\frac{1}{2}$ nuclei of hydrogen (^1H). In the case of spin- $\frac{1}{2}$, the spins are either parallel or anti-parallel to the external field, and the lowest state is with the spins parallel to the applied field, similar to how a bar magnet aligns parallel to an external magnetic field (Fig. 1.1). Note that the bar magnet analogy is a simplified interpretation of the quantum mechanical nature of spin. In fact, spin can exist in any linear combination of the $+\frac{1}{2}$ and $-\frac{1}{2}$ states. For a detailed description, see Levitt (2001).

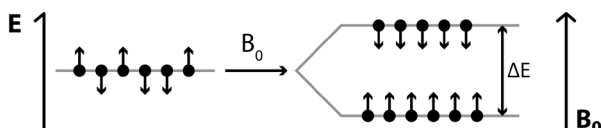


Fig. 1.1 Orientation of spin in spin- $\frac{1}{2}$ nuclei, in response to an external static magnetic field (B_0). In the initial state, the spins are randomly distributed over the $+\frac{1}{2}$ and $-\frac{1}{2}$ states. When the magnetic field is applied, the nuclei orient themselves in one of two possible directions: parallel to the magnetic field, or antiparallel. The parallel orientation is very slightly more energetically favoured than the antiparallel state. This minute difference means slightly more spins are oriented parallel to the field. Note that this model is a simplified interpretation of the quantum reality of spin.

There is a slight preponderance for spins to align parallel to the field, which is crucial as this difference can be used to excite to an observable magnetisation. The energy difference depends on the magnet field strength B_0 . (Eq. 1.1).

$$\Delta E = \gamma \hbar B_0 \quad (1.1)$$

Where $\hbar$ is the reduced Plank constant ($h/2\pi$), and γ is the gyromagnetic ratio of the specific nucleus in question. The ratio of parallel vs anti-parallel spins follows the Boltzmann distribution (Eq. 1.2):

$$\frac{N_{ap}}{N_p} = e^{-\frac{\Delta E}{kT}} \quad (1.2)$$

Where N_{ap}/N_p is the ratio of parallel vs anti-parallel spins, ΔE represents the energy difference described by Eq. 1.1, T is the temperature in Kelvin, and k is the Boltzmann constant.

The excess spins in the parallel state represent a small fraction, roughly around 1 in 10^4 spins under an ultra-high field strength of 22.3 T. This small polarisation means that NMR is an inherently low sensitivity technique, but due to the considerable amount of spins in a typical sample, there is still sufficient signal to detect. The magnetisation can be represented by a vector, containing both magnitude and direction of the magnetisation. By convention, the direction of the magnetic field lies along the Z-axis.

The second analogy is that of a spinner toy. Normally, when spun, a spinner will try to remain upright as it tries to retain angular momentum. When the spinner is perturbed, for example by a gentle nudge, its angular momentum gives rises to an unusual phenomenon called precession. Precession is the tendency to resist changes to an object's momentum by rotating around its axis in a manner depicted in Fig. 1.2A. Nuclei with spin react in the same way as a spinner or gyroscope when forced out of balance; they too will precess. When discussing precession, it is easier to use a rotating frame of reference, where the X and Y axes spin at the same frequency (ω) as the vector of interest. A vector with the same precession frequency (ω) appears to be static in the rotating frame (Fig. 1.2B).

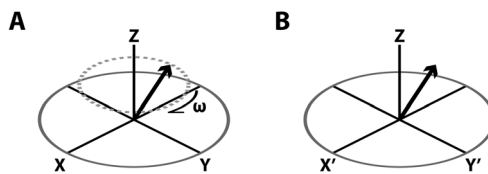


Fig. 1.2 The precession phenomenon. (A) A vector is used to represent precession with an arbitrary frequency (ω) around the z-axis. (B) A useful tool to work with precession is the rotating frame, where the X-axis and Y-axis rotate at the same frequency (ω) as the precession of interest. The rotating frame axes are denoted with X' and Y' respectively. The rotating frame results in a vector that appears static in the rotating frame.

1.1.1 MAGNETISATION

In summary, spin combines some properties of angular momentum and magnetism. The polarisation due to excess spins in the parallel orientation (Z-direction) along a magnetic field, results in a magnetisation that can be represented as a vector. If the spins are perturbed, this vector will start to precess in the XY-plane (Fig. 1.2). It turns out that the precession frequency (ω) depends on the strength of the magnetic field, which is described by the Larmor equation (Eq. 1.3):

$$\omega = \gamma B_0 \quad (1.3)$$

For ^1H , the gyromagnetic ratio (γ) is 42.57 MHz T^{-1} , which is the highest of all nuclei, and B_0 is the magnetic field strength in Tesla. For a 17.6 T magnet, this results in a resonance frequency of 750 MHz. Generally, superconducting magnets are used to achieve high field strengths. Conveniently, the natural abundance of hydrogen is almost 100%, which combined with the high concentration of hydrogen nuclei in biological tissues, means hydrogen is the predominate nucleus used for NMR (Plewes and Kucharczyk, 2012).

In order to perturb or excite the spins, a secondary transient magnetic field in the rotating frame is used. This magnetic field can be generated with a radiofrequency (RF) pulse at the resonant frequency, *e.g.*, 750 MHz. The RF pulse flips the magnetisation to the XY- or transverse plane. The flip angle (α) can be controlled by varying the duration of the RF pulse (Eq. 1.4).

$$\alpha = \gamma B_1 \tau \quad (1.4)$$

Where τ is the duration of the pulse and B_1 is RF pulse field strength.

Using RF receiving coils, also called resonators, the magnetisation in the transverse plane can be detected, and consequently, Fourier transformed to analyse the sample. Manipulating nuclear spin magnetisation with RF pulses is the basis of nuclear magnetic resonance (NMR) and gives rise to a wide variety of pulse schemes to investigate various aspects of samples of interest.

1.1.2 LONGITUDINAL AND TRANSVERSE RELAXATION

Immediately after a sample is excited using an RF pulse, it will start to relax back to its equilibrium state with all the magnetisation pointing along the z-direction. This return to equilibrium is called longitudinal relaxation. The decay of magnetisation is exponential and governed by equation 1.5:

$$M_z(t) = M_{z,0} \left(1 - e^{-\frac{t}{T_1}} \right) \quad (1.5)$$

Where $M_{z,0}$ is the equilibrium state, and T_1 controls the rate of decay. T_1 is defined as the point in time where e^{-1} or ~63% of magnetisation has relaxed to equilibrium (Fig. 1.3A).

The evolution of magnetisation following an initial 90° RF pulse is shown graphically in Fig. 1.3B.

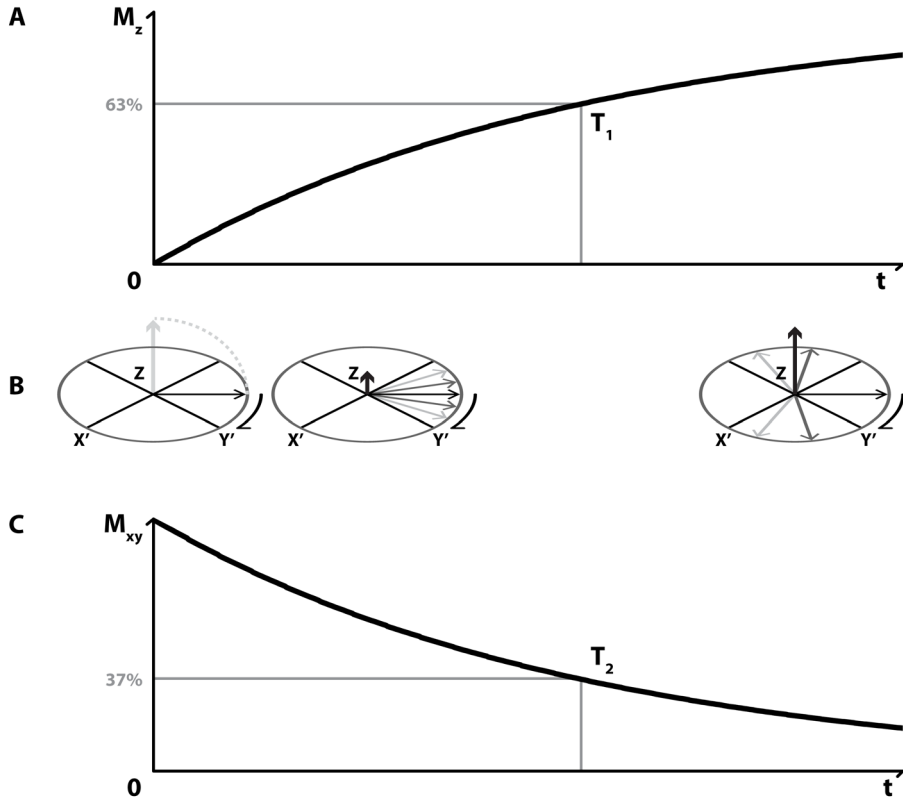


Fig. 1.3 T_1 and T_2 relaxation. (A) Longitudinal or T_1 relaxation. After an initial 90° excitation pulse, longitudinal magnetisation recovers exponentially. At T_1 , approximately 63% of M_z magnetisation has recovered. (B) Graphical representation of T_1 and T_2 relaxation. The initial state depicts a 90° pulse flipping full magnetisation in the transverse (XY) plane. Next, the resonance evolves progressively until near complete relaxation. A thick black vector indicates longitudinal magnetisation, while arrows in the XY-plane depicts transverse dephasing due to different resonant frequencies. (C) Graph of transverse or T_2 relaxation. Due to differences in resonance frequencies, over time spins dephase which destroys the coherence of the signal. Similar to T_1 , T_2 is defined as the time were 37% of the transverse magnetisation remains.

A second mechanism termed transverse or T_2 relaxation causes loss of magnetisation in the XY-plane. Transverse relaxation is due to spin dephasing, caused by a difference in resonant frequency of the spins in the sample, see Fig. 1.3B. Because individual spins cannot be detected, only the summation of all spins, loss of coherence results in loss of detectable signal. Like longitudinal relaxation, transverse relaxation is an exponential process:

$$M_{XY}(t) = M_{XY,0}e^{-\frac{t}{T_2}} \quad (1.6)$$

Where $M_{XY,0}$ is the magnetisation immediately after excitation, and T_2 is the duration of time when e^{-1} or 63% of total magnetisation has been relaxed (Fig 1.3C). Local field inhomogeneity caused by imperfections in the B_0 field or local differences in susceptibility, *e.g.*, air-water transitions, can cause the signal to relax faster than predicted by Eq. 1.6. The actual observed T_2 is termed T_2^* . Some strategies are used to mitigate signal loss due to T_2^* effects, such as the spin echo sequence. In spin echo sequences the evolution of spins is reversed by applying a 180° pulse. This 180° pulse reverses the direction of all spins, to refocus the T_2^* induced changes.

Both T_1 and T_2 relaxation occur simultaneously, but typically the T_1 is much longer (in the order of seconds) than the T_2 (in the order of milliseconds).

1.1.3 MAGNETIC RESONANCE IMAGING: SPATIAL ENCODING

When an RF pulse excites a sample within an NMR instrument, typically a signal arising from the entire sample is detected. If one wants to use the NMR phenomenon to create images, this signal needs to be localised. Magnetic Resonance Imaging (MRI) is able to achieve localisation by employing transient magnetic fields, whose strength varies depending on location. These magnetic fields are generated by supplying current to loops of wires that can generate linear magnetic field gradients in three orthogonal directions (X, Y, Z). These fields result in the resonance frequency becoming linearly dependent on the position of the nuclei within the sample holder. For successful imaging, the signal has to be localised in all three dimensions. Three approaches may be used to achieve this, generally in the following order: slice selection, phase encoding and frequency encoding.

Slice selection is often used as the first step in localising the NMR signal. Simultaneously with an RF pulse, a magnetic gradient is applied, resulting in the selection of a slice of spins within the sample. Figure 1.4A indicates the overlap of RF bandwidth and the frequency range within the sample.

Encoding of the second dimension is done using phase encoding. By applying a magnetic gradient along, for example, the Y-axis, spin precession speeds up or slows down, depending on the location of the spins in the magnetic field (Fig. 1.4B). When the magnetic field gradient is released, the spin precession frequencies return to normal, but the phase of the spin will either lead or trail the reference spin phase at the centre of the coils. Thus information on the location of spins in the Y-dimension has been encoded in their phase angle. For each line in an image, the strength of the phase encoding gradient may be increased.

Spatial information on the last dimension can be obtained through frequency encoding (Fig. 1.4C). The signal is recorded or ‘sampled’ multiple times during signal acquisition, while a gradient is active in the Z-dimension. The frequency of spins within the sample

thus continuously evolves between each sampling point. The rate of change of spins between these sampling points then reveals the position of the spins along the Z-axis.

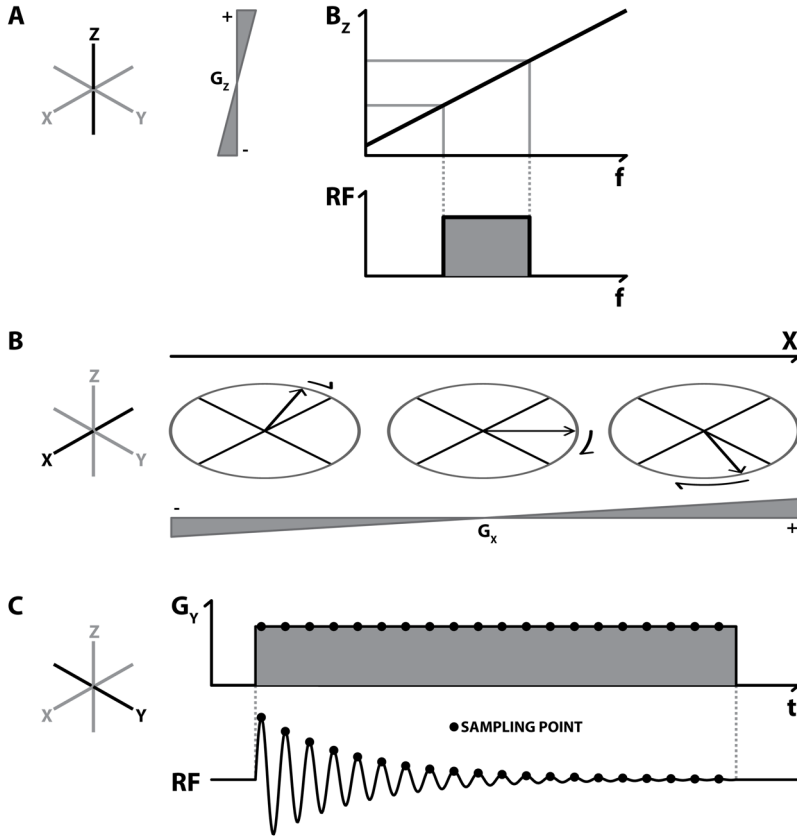


Fig. 1.4 Spatial encoding techniques of MRI. (A) Slice selection is achieved by applying a magnetic field gradient (shown here for the Z-axis), which makes the resonant frequency dependent on the position along the Z-axis. Simultaneously, an RF pulse is given, resulting in the excitation of spin polarisation with frequencies matching the RF pulse bandwidth. Thus a slice of spins is selected within the sample. (B) Phase encoding occurs using a gradient in an orthogonal direction to that of the slice selection, here the X-axis. This results in spin frequencies increasing or decreasing depending on their location. Once the gradient ends, the temporary frequency differences are retained as corresponding differences in the phase of spins. The phase encoding is varied for each line of an image. The strength of phase encoding depends on the strength and duration of the gradient. (C) Frequency encoding occurs when a signal is sampled multiple times while a gradient is active along the Y-axis. The gradient causes the frequency to evolve continuously while the signal is sampled. The localisation of the signal may then be reconstructed using Fourier transforms on the phase and frequency encoding directions (Plewes and Kucharczyk, 2012).

1.1.4 LOCALISED SPECTROSCOPY: VOLUME SELECTION

A localised NMR spectrum may be acquired by successive application of slice selection in all three dimensions. When implemented as a spin echo sequence, based on pulses

with a 180° flip angle, magnetisation is refocused in a three-dimensional volume of interest (VOI) at the intersection of three slice selective pulses.

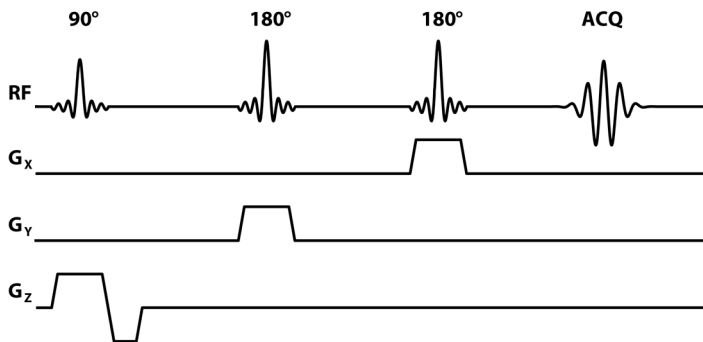


Fig. 1.5 Pulse sequence diagram of Point RESolved Spectroscopy (PRESS). A 90° - 180° - 180° pulse train coupled with simultaneous mutually orthogonal gradients selects a rectangular volume of interest. A localised spectrum is then obtained (Bottomley, 1987).

Figure 1.5 shows a pulse sequence diagram where each gradient is applied in a different plane, thus selecting a VOI. Only the VOI experiences all three (re)focusing pulses, all other areas receive only one or two pulses and are thus dephased by the time of signal acquisition. While PRESS results in a 1D spectrum, originating from the VOI, its volume selecting properties may also be used as the basis for more advanced pulse sequences.

1.1.5 DIFFUSION WEIGHTED IMAGING

MRI may also be used to investigate diffusion occurring within biological tissue (Luytjens *et al.*, 2001). Spatial encoding depends on the assumption that the nuclei-containing molecules remain static. This assumption, however, breaks down on a microscopic level because the molecules containing spins are subject to Brownian motion and depending on their local environment, free to diffuse (Le Bihan *et al.*, 2006). The assumption also breaks in the case of convection within samples, *e.g.*, transport of fluids in veins and channels. These phenomena can be used to reveal valuable information. In the case of diffusion, one can dephase spins using a gradient, and after an evolution time to allow for diffusion, rephase spins with an inverse gradient (Fig. 1.6A). For spin echo sequences, a Stejskal-Tanner type sequence with a gradient on either side of a 180° pulse is often used (Stejskal and Tanner, 1965). If a molecule experiences little diffusion, it will be in the same VOI when the second gradient is applied (Fig. 1.6B). The amount of phase encoding is, therefore, the same and its signal is not attenuated. However, a molecule that experiences strong diffusion will have moved in the time (Δ) between the two gradients. Since the molecule has moved, it will experience a different gradient strength in its new location. The result is a net signal attenuation that depends proportionally on the distance travelled.

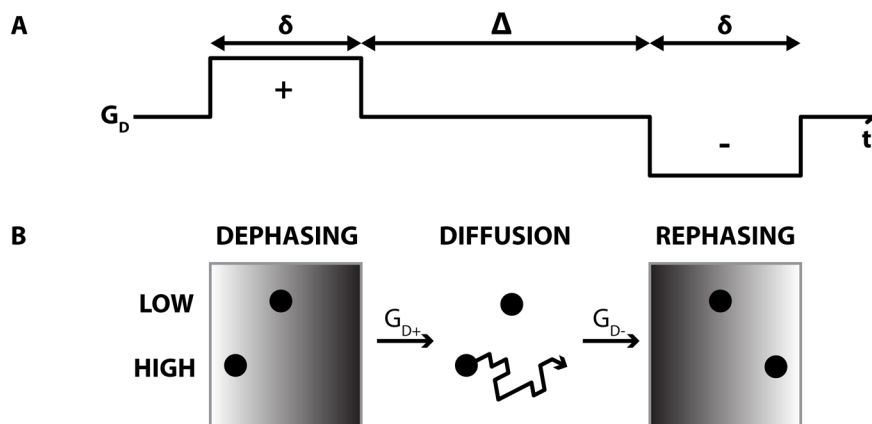


Fig. 1.6 Diffusion weighting using twin diffusion sensitising gradients. (A) A pair of gradients with the same strength and duration (δ), but inverse polarity, is used to attenuate the signal from molecules exhibiting high rates of diffusion selectively. The strength of the diffusion encoding depends on the strength and length of the gradients as well as an evolution time (Δ) between the gradients. (B) Graphical representation of diffusion. A particle with little diffusion moves a negligible amount between the two gradient pulses. Since it experiences the same amount as dephasing and rephasing, no signal attenuation takes place. In contrast, a high diffusion particle will move in between the two sensitising gradients and thus experience a net positive amount of dephasing. This dephasing results in signal attenuation that may be used to calculate an apparent diffusion coefficient (ADC).

An apparent diffusion coefficient (ADC) can be calculated using two or more images acquired with different diffusion weighting. The ADC is useful since it is a global statistical parameter that can be readily calculated for experimental measurements, in contrast to the underlying physical diffusion parameter D , which is difficult to determine experimentally (Le Bihan *et al.*, 2006). The ADC depends on experimental conditions such as voxel size or gradient duration, which can make it difficult to compare across different experiments. The strength of the diffusion weighting may be expressed as B-values ($s\ mm^{-2}$). The B-value is a convenient measure to express the total strength of the diffusion encoding experienced by a given molecule during the experiment. High B-values may be attained by strong gradients, long gradient durations or long gradient separation duration, or any combination thereof.

1.1.6 DIFFUSION WEIGHTED CHEMICAL SHIFT IMAGING DW-CSI

Several of the techniques described here may be combined in a modular approach that results in a sequence combining spatial localisation of spectroscopic information with diffusion weighting. The resulting sequence, Diffusion Weighted Chemical Shift Imaging (DW-CSI), is voxel-based with a 90° - 180° - 180° PRESS pulse train, see Fig. 1.7 (Ercan *et al.*, 2015). A slice selective gradient accompanies each radiofrequency (RF) pulse. Here shown as G_z for the 90° pulse. Each consecutive 180° pulse has a slice selective gradient applied in an orthogonal direction, G_y or G_x .

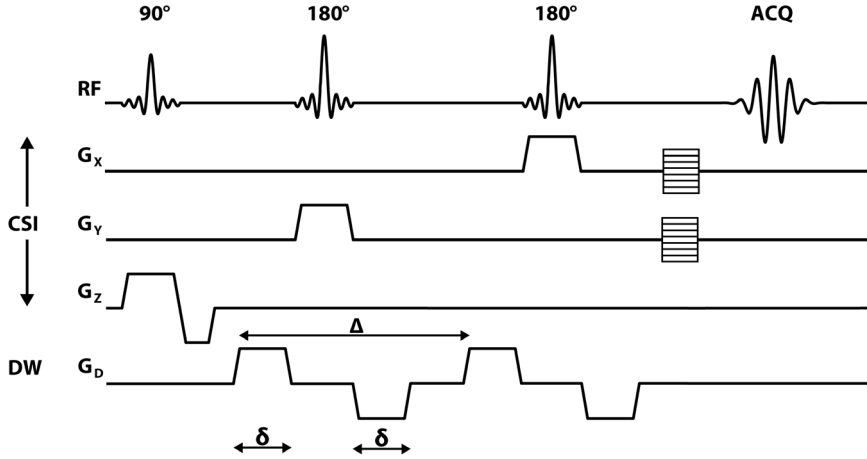


Fig. 1.7 DW-CSI Sequence Diagram. Radiofrequency pulses and resulting echo are shown on the top line. Chemical shift encoding is performed by a series gradients, shown here with G_z fixed and increments in G_x and G_y for each matrix position. Diffusion gradient, G_D , is shown separate from the CSI gradients, but in practice is intertwined with $G_{x,y,z}$ in any direction chosen at will. Diffusion sensitising strength is determined by the gradient separation (Δ), multiplied by the gradient duration (δ) and gradient strength.

The net result is efficient refocusing of magnetisation as an echo during acquisition (ACQ), within the intersection of slice gradients. The gradient directions may be chosen freely, so long as the gradients are mutually orthogonal. In contrast to regular imaging, CSI omits the readout gradient found in typical imaging sequences. Diffusion weighting is incorporated into the sequence by applying bi-polar diffusion sensitising gradients, centred around the two 180° pulses. The bi-polarity helps to avoid cross terms between the localising gradients and the diffusion sensitising gradients. For ease of comprehension, the diffusion weighting gradients (G_D) have been depicted separately from the main CSI sequence (Fig. 1.7). The diffusion gradients are interleaved with the main CSI sequence and can be applied in any primary direction or mixture thereof. For the sake of simplicity, the VAPOR water suppression immediately preceding the sequence has also been omitted.

1.1.7 MAGNETIC RESONANCE MICROSCOPY

Magnetic Resonance Microscopy (MRM) is defined as imaging at resolutions below 100 μm (Glover and Mansfield, 2002). High-resolution imaging requires high sensitivity since the experimental signal-to-noise ratio (SNR) scales linearly with the voxel volume, *i.e.*, $\text{SNR} \propto 1/D_{\text{voxel}}^3$. An increase in resolution from 20 μm isotropic resolution ($20 \times 20 \times 20 \mu\text{m}^3$) to 10 μm isotropic resolution would decrease the voxel SNR by a factor 8. If one wishes to retain the same SNR as the lower resolution image, one could increase the number of measurements, since the SNR scales with the root of the number of experiments ($\text{SNR} \propto \sqrt{N_{\text{exp}}}$). Thus, measurement time would need to be 64 times longer, which illustrates the importance of maximising the SNR via other means.

In order to maximise the experimental SNR, it is useful to investigate the contribution of different factors with a simplified equation for the SNR expressed in observed voltage (G. Webb, 1997; Webb, 2012):

$$SNR \propto [\gamma B_0] \left[\frac{\gamma^2 h^2 B_0 N_s}{16\pi^2 k T_s} \right] \left[\frac{B_1}{I} \right] \left[\frac{1}{V_{\text{noise}}} \right] \quad (1.7)$$

Here h is Plank's constant, N_s is the number of spins per volume, k is Boltzmann's constant, T_s is the sample temperature, B_1/I is the magnetic field strength generated per unit current, V_{noise} is the total voltage noise, including sample and circuitry noise. The simplified equation may be broken down into four terms. The first term describes the relation of the induced voltage to the magnetic field flux (B_0). The second term describes the total magnetisation within the sample, which depends on the number of spins (N_s), field strength and temperature of the sample. Both the first and second term depend on B_0 . SNR scaling with respect to B_0 has been reported in the literature as $B_0^{3/2}$ or $B_0^{7/4}$ (Hoult and Richards, 1976; Nakada, 2007; Webb, 2012). This dependence has driven the development of increasingly high field strengths, with pre-clinical MRI instruments of up to 22.3T in use. However, under ultra-high field conditions, the experimentally measured SNR correlation is approximately linear (Schepkin, 2016). The third term, B_1/I , describes the relation of SNR to the coil sensitivity, which has resulted in the creation of a broad range of coil geometry types and optimised coil designs (Fratila and Velders, 2011). Lastly, the fourth term, V_{noise} corresponds to the noise voltage, which itself consists of:

$$V_{\text{noise}} = \sqrt{4kT_c \Delta f R} \quad (1.8)$$

Where T_c is the temperature of the RF coil, Δf is the receiver bandwidth, and R is the resistance of the coil. The temperature dependence means that the noise voltage may be reduced by cooling the resonator and pre-amplifiers. In particular, cooling of both RF coils and pre-amplifier in cryoprobes results in SNR gains with a factor 3-4 (Kovacs, Moskau and Spraul, 2005). The insulation required between the sample, the coil and the instrument, poses significant technical challenges, increasing the complexity of cryogenic coil designs (Koo *et al.*, 2011).

MRM is made possible by using the three approaches of increasing field strength, optimised coil design and cooling RF circuitry, either separately or in conjunction with each other. Here, we focus on miniaturised coils or microcoils for use in ultra-high field spectrometers.

1.1.8 MICROCOILS

The highest possible image resolutions (up to 3 μm isotropic) have been attained with very small resonators called microcoils, with diameters (D_{coil}) in the mm to μm range (Ciobanu, Seeber and Pennington, 2002; Weiger *et al.*, 2008). Microcoils owe their sensitivity to the inverse scaling of SNR with coil diameter. The SNR of a solenoid

resonator increases as the diameter decreases with $1/D_{\text{coil}}$. Below roughly 1mm in coil diameter, the sensitivity of microcoils scales with $1/\sqrt{D_{\text{coil}}}$ (Peck, Magin and Lauterbur, 1995; Glover and Mansfield, 2002). Thus, coil design must closely match the sample size to ensure the highest achievable sensitivity. The ratio between sample volume and coil volume is termed the coil filling factor (η).

Several types of coil design exist, including Helmholtz, saddle, birdcage, planar, stripline and solenoid coils design (Fratila and Velders, 2011). Solenoid coils are used extensively for MRM since they combine high sensitivity, homogeneous B_1 fields and ease of construction. The solenoid microcoil consists of a conducting wire wrapped around a capillary in the form of a helix (Fig. 1.8). The solenoid wire may be wound by hand, and the circuitry can be hand-soldered. Due to these properties, solenoid coils were selected for use in this thesis.

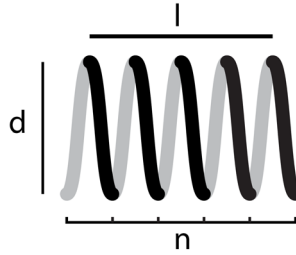


Fig. 1.8 Diagram of the solenoid coil and its measures. The dimensions of a solenoid coil are determined by its diameter (d), length (l) and the number of turns (n).

Like all NMR coils, microcoils operate by the principle of induction. The NMR phenomenon induces a voltage oscillation in a resonant circuit, consisting of the coil and a capacitor. Though radiofrequency pulses are used, NMR is not a radiofrequency spectroscopic technique since the signal observed in detection coils is caused by induction, with radio waves playing a negligible role (Hoult, 2010).

The sensitivity per unit of current (B_1/I) of a solenoid resonator may be approximated with the following equation (Minard and Wind, 2001) :

$$\frac{B_1}{I} = \frac{\mu n}{d \sqrt{1 + \left(\frac{l}{d}\right)^2}} \quad (1.9)$$

Where μ_0 is the permeability of free space, n is the number of turns, d is the diameter of the coil and l is the length of the coil. If the ratio of length over diameter is kept constant, the sensitivity increases linearly as the coil diameter decreases. A higher number of turns also increases sensitivity, but the number of turns must be reduced as B_0 increases in order to avoid self-resonance on frequencies that interfere with the coil operating

frequency (Subramanian, Lam and Webb, 1998). The resonance frequency depends not only on the geometry of the coil, which determines the inductance, but also the capacitance of the circuit. In its simplest form, a resonator consists of an inductor (L) placed in parallel with a capacitor (C) to form a circuit (Fig. 1.9).

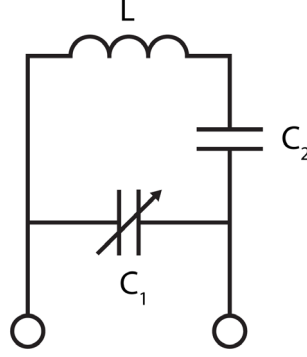


Fig. 1.9 Electrical Diagram of solenoid microcoil. The windings of the solenoid coil function as an inductor (L), while capacitors C_1 and C_2 are placed in series, parallel to the coil. C_1 often has a variable capacitance to allow for tuning.

The conditions for resonance are met if the reactance of L and C are equal (Wheeler and Conradi, 2012). Since inductive reactance increases with frequency while capacitive reactance decreases with frequency, there is a resonant frequency where inductance and capacitance are equal (Eq. 1.10):

$$\frac{\omega^2 LC}{2\pi} = 1 \quad (1.10)$$

Which leads to the resonance frequency:

$$\omega = \frac{1}{2\pi\sqrt{LC}} \quad (1.11)$$

By choosing an appropriately sized capacitor (C_1), the resonant frequency may be adjusted to obtain the required operating frequency. If a variable capacitor is used for C_1 , it may be used for tuning of the resonant frequency. A capacitor (C_2) is placed in series with the coil, allowing the microcoil to be matched to 50Ω by reducing the impedance of the circuit (Subramanian, Lam and Webb, 1998).

1.2 BIOFUELS DERIVED FROM ALGAE: *B. BRAUNII*

In the context of renewable energy, biodiesel is often considered, due to its potential to replace fossil fuels without major changes to fuel infrastructure or engines, for instance

in aviation (Kandaramath, Yaakob and Binitha, 2015; Yilmaz and Atmanli, 2017). Biodiesel may be derived from a variety of biomass sources, but algae are of particular interest due to their lack of competition with food production since no arable land is required for cultivation (Chisti, 2008; Benemann, 2013).

Botryococcus braunii has a global distribution and is found in brackish and freshwater environments (Wake and Hillen, 1980; Aaronson *et al.*, 1983; Metzger and Largeau, 2005). The high fraction of hydrocarbons present in *Botryococcus braunii* has attracted much attention, as they can be transformed into biodiesel (Hillen *et al.*, 1982; Eroglu and Melis, 2010; Demirbas and Fatih Demirbas, 2011). *B. braunii* accumulates high concentrations of oil as part of its normal life cycle, which may be improved by changing (stress) conditions, *e.g.* nitrogen deficiency (Al-Hothaly *et al.*, 2016; Cornejo-Corona *et al.*, 2016). Because of the relevance to biofuels, considerable effort has been spent on characterising *B. braunii* oils (Metzger *et al.*, 1985; Huang and Poulter, 1989; Sato *et al.*, 2003). Several strategies for oil extraction are currently available, with new possibilities being sought to extract oil without compromising colony viability (Furuhashi, Hasegawa, *et al.*, 2016). Attention has been given to the interaction of solvents with the colony exterior (Furuhashi, Noguchi, *et al.*, 2016). The lack of success in commercialisation of biofuels derived from *B. braunii* is attributed to its slow growth (Cabanelas *et al.*, 2015; Gouveia *et al.*, 2017). The slow growth itself may be correlated with the accumulation of hydrocarbons due to negative feedback (Banerjee *et al.*, 2002). More detailed understanding of the influence of oil on colony structure and growth is therefore desirable.

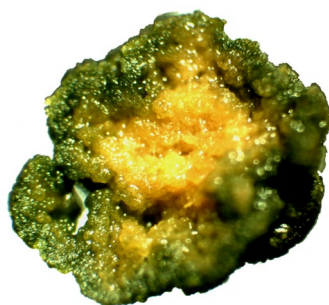


Fig. 1.10 Optical microscopy of a single *Botryococcus braunii* var. *showa* colony. The colony has been cut across, revealing the interior that displays an orange hue due to carotenoids. The background has been digitally removed. Image reproduced courtesy of Tomas van der Berg.

Botryococcus braunii exhibits a range of different metabolites depending on its race and strain. Here, the Race B variety *showa* (Berkeley strain) is studied exclusively (Fig 1.10). Since oil plays a central role in the morphology of *B. braunii*, we are interested in its influence on the diffusion of metabolites within the colony. MRI is used to study whole colonies non-invasively, combining imaging of their structure with metabolite mapping and diffusion measurements.

1.3 ROOT NODULATION IN *MEDICAGO TRUNCATULA*

Nitrogen plays a central role in biological processes, being a crucial component of DNA, RNA and the amide backbone of proteins. Bio-availability of nitrogen is, therefore, an essential factor determining the growth rate of plants. Nitrogen is mainly present in the atmosphere in its gaseous form (N_2), in which a strong triple bond binds two nitrogen atoms. Plants can only utilise nitrogen in organic forms, but the strength of the triple bond makes it energetically costly for plants to fix nitrogen from the air. Instead, through a remarkable feat of co-evolution, plants have effectively outsourced nitrogen fixation to a collection of metabolic symbionts, which includes arbuscular mycorrhizae and rhizobacteria (Bonfante and Genre, 2008; Suzaki, Yoro and Kawaguchi, 2015). These symbionts provide the plant with fixed nitrogen in return for nutrients such as sugars (Pfau *et al.*, 2016).

The mutualistic interaction with organisms from the genera Rhizobacteria is among the most intricate forms of symbiosis, in which leguminous plants have evolved root nodules that host the bacterial partners and facilitate nutrient exchange (Suzaki, Yoro and Kawaguchi, 2015). These organs grow in response to signalling from bacterial partners present in the rhizosphere, with each plants species having specific rhizobial partners (Xiao *et al.*, 2014). These mutualistic metabolic interactions also confer plants with increased resistance against drought and other stress conditions (Kunert *et al.*, 2016; Staudinger *et al.*, 2016; Vurukonda *et al.*, 2016). Legumes are amongst the most important agricultural crops, including *Glycine max* (soybean) and *Medicago sativa* (alfalfa), since they can replenish organic nitrogen in soils and can improve soil health through their microbial partners (Doran and Zeiss, 2000). Agricultural practices that make use of legumes also reduces reliance on artificial fertilisers. A deeper understanding of the metabolic interactions within the root nodule of legumes could improve the sustainability of agricultural practice.

Since roughly half a plants biomass resides underground, it can be difficult to study without disturbing the plant or rhizosphere. MRI allows to study root systems of live plants non-invasively, and also allows for longitudinal experiments (Ishida, 2000; Van As and van Duynhoven, 2013). Applications include tracking root growth and 3D reconstructed models of root systems (Metzner *et al.*, 2014; Schmittgen *et al.*, 2015; van Dusschoten *et al.*, 2016). Gas exchange in root nodules of *G. max* has also been studied (Macfall *et al.*, 1992; Brown *et al.*, 1997; Chudek *et al.*, 1997). Here, we study root nodules of *Medicago truncatula*, a model system for root nodulation in legumes which belongs to the same genus as *Medicago sativa* (Barker *et al.*, 1990). Dedicated microcoils for MRM

were developed to obtain *in situ* anatomical images with cellular resolution as well as localised metabolic profiles. MRM and MRS results were then cross-referenced with optical microscopy.

1.4 THESIS SCOPE

Central to the scope of this thesis is the development of MRI applications in *B. braunii* and algae, to answer questions related to food and energy sustainability. To advance the possible applications of MRI in these topics and specimens, a threefold strategy is used. First is the optimisation of pulse sequences on these challenging specimens, including imaging and chemical shift selective pulse sequences, as well as diffusion weighting. Secondly, to develop custom hardware dedicated to specific samples, in the form of microcoils at high and ultra-high field, enabling imaging at cellular resolution. Lastly, by adapting the latest in pulse-sequence designs to microimaging.

In **Chapter 2**, the localisation of hydrocarbons and oils in *B. braunii* var. *showa* is discussed, as well as the two types of colonies which were observed. Diffusion and presence of water channels has been visualised for the first time. In **Chapter 3**, application development for microcoils is examined, and a methodology for testing novel microcoils is introduced. **Chapter 4** presents a practical application of microcoils to study the metabolic profile of *Medicago truncatula* root nodules infected with *Sinorhizobium meliloti*. In **Chapter 5**, the future of micro-imaging and combining diffusion encoding and chemical shift imaging into a DW-CSI is examined, and the potential development of advanced microcoil design is discussed.

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2 NON-INVASIVE MR IMAGING OF OILS IN *B. BRAUNII* GREEN ALGAE

Chemical Shift Selective and Diffusion-Weighted Imaging

2.1 ABSTRACT

Botryococcus braunii is an oleaginous green algae with the distinctive property of accumulating high quantities of hydrocarbons per dry weight in its colonies. Large variation in colony structure exists, yet its implications and influence of oil distribution and diffusion dynamics are not known and could not be answered due to lack of suitable *in vivo* methods. This chapter seeks to further the understanding on oil dynamics, by investigating naturally relevant large (700-1500 μm) and extra-large (1500-2500 μm) sized colonies of *Botryococcus braunii* (race B, var. *showa*) *in vivo*, using a comprehensive approach of chemical shift selective imaging, chemical shift imaging and spin echo diffusion measurements at high magnetic field (17.6 T). Hydrocarbon distribution in large colonies was found to be localised in two concentric oil layers with different thickness and concentration. Extra-large colonies were highly unstructured and oil was spread throughout colonies, but with large local variations. Interestingly, fluid channels were observed in extra-large colonies. Diffusion-weighted MRI revealed a strong correlation between colony heterogeneity, oil distribution, and diffusion dynamics in different parts of *Botryococcus* colonies. Differences between large and extra-large colonies were characterised by using T_2 weighted MRI along with relaxation measurements. Our result, therefore provides first non-invasive MRI means to obtain spatial information on oil distribution and diffusion dynamics in *Botryococcus braunii* colonies.

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2.2 INTRODUCTION

Research into biofuel sources is receiving increasing attention as the general public and policymakers become aware of the need to shift from a fossil energy based economy to a more sustainable bio-based economy. The search for new fuels is driven in part by the predicted economic consequences of climate change, but also by the necessity of replacing finite resources (IPCC and Core Writing Team, 2014). However, first and second generation biofuels have difficulty reaching sufficient economic efficiency, due to the costly conversion steps involved, energy diverted to biomass and a large areal footprint. Therefore, third generation biofuels ideally need to provide direct conversion of CO₂ into biofuels, avoid conversion losses and also utilize biofuels as an energy sink which would altogether increase yield. Algae, known for their large biodiversity and range of secondary metabolites, could provide a promising solution for this challenge.

Algae-derived biomass has already been suggested as a possible aqua-based alternative to land-based crops (Demirbas and Fatih Demirbas, 2011). More specific, green algae such as *Botryococcus braunii*, (*B. braunii* var. *showa* (Nonomura, 1988)) have the advantageous property that they produce oils in lipid bodies, mainly C₃₀ to C₃₄ botryococcenes, like showacene and isoshowacene (Wolf, Nonomura and Bassham, 1985; Huang and Poulter, 1989). Hydrocarbons are present in the form of oils that are similar to those found in petrochemical sources and can be readily refined using hydrocracking (Hillen *et al.*, 1982). Algaenane complexes comprised of a variety of polymethylated squalenes are also present in *B. braunii* race B (Metzger, Rager and Largeau, 2007). In addition, *B. braunii* is considered to be an important contributor to petroleum generation, being linked to Torbanite and Coorongite oil shales (Dubreuil *et al.*, 1989; Glikson, Lindsay and Saxby, 1989; Kumar *et al.*, 2016).

Oil in *Botryococcus* is believed to serve multiple purposes, including buoyancy control that allows for floatation (Glikson, Lindsay and Saxby, 1989; Eroglu, Okada and Melis, 2011). Furthermore, fatty acids excreted by some strains have allelopathic effects on other phyto algae and cause fish death during blooms (Chiang, Huang and Wu, 2004). Much attention has been focused on improving the relatively slow growth of *Botryococcus* strains (Yoshimura, Okada and Honda, 2013; Jin *et al.*, 2016). Hydrocarbon accumulation is intimately linked to cell division, being important for cell-cell cohesion and structure, thus influencing growth patterns (Tanoi, Kawachi and Watanabe, 2014). Recent work on *B. braunii* Race A indicates that lipid bodies are formed in the cytoplasm, with hydrocarbon synthesis reaching its maximum during septum formation (Hirose *et al.*, 2013). Suzuki *et al.* proposed the central role of outer cell wall layers in the formation of an intercellular matrix, formed by successive cell divisions (Suzuki *et al.*, 2013). These findings, combined with accumulation of oil in the extracellular spaces, have captivated research attention focused on using *B. braunii* for biofuels for decades, with publications starting to accumulate from the 1980's onwards. The interest increases explosively from the 2000's up to now.

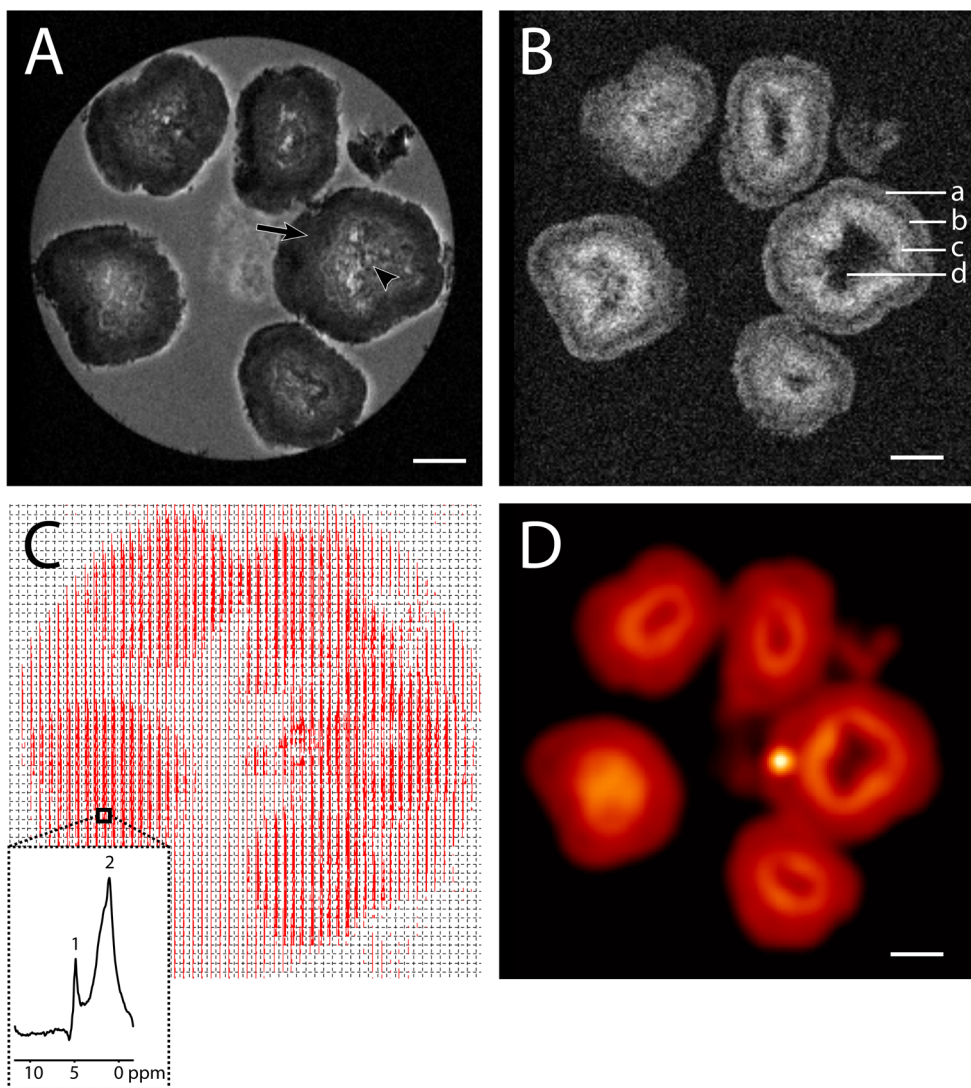
Until now, detailed information on colony anatomy is restricted to small sized colonies (30-200 μm) [16,18,19]. However, *B. braunii* shows a large diversity in colony size under natural conditions, with values of 30-2000 μm being reported in the wild (Rivas, Vargas and Riquelme, 2010). There also exist naturally occurring algal blooms with colony sizes of up to 1500 μm (Wake, 1983). Bloom formation is especially notable in water reservoirs where blooms were reported up to 1500 metric tonnes in terms of biomass (Wake and Hillen, 1980; Wake, 1983). A detailed and comprehensive picture of *B. braunii* physiology and its oil accumulation characteristics under natural conditions is still missing. It is unknown how the large variation in the colony size is linked to oil accumulation in localized domains and whether diffusion characteristics are influenced by colony structure. Observing the anatomical structure of various colonies, together with direct *in vivo* mapping of oil domains, would help us to understand the link between colony structure and oil accumulation behaviour. These observations could provide insight into the functions and mechanisms underlying these large variations in colony structure. Furthermore, distribution of different types of oil within larger sized colonies could be useful for the prediction and optimisation of production yields. The unique properties of *B. braunii* make experimental studies challenging, especially considering the copious mucilage exuding from the colonies and the large range in colony size (Wake, 1983). Optical microscopy, including staining, FLIM, etc., allow for high-resolution study of colony anatomy but relies on invasive cross sections, and the metabolite composition in localized domains within intact colonies cannot be approached. Solution state NMR and HR-MAS NMR have been utilized to determine lipid contents extracted from *B. braunii* colonies (Simpson *et al.*, 2003; Ruhl, Salmon and Hatcher, 2011). However, localized information about lipid and metabolite distribution and their relation to colony structure cannot be obtained in intact live colonies utilising these techniques. Among other strategies to overcome these limitations, confocal Raman spectroscopy has seen application in *B. braunii* race B to image specific hydrocarbons, although with low resolution (Weiss *et al.*, 2010).

This study, therefore, aims to optimize and exploit non-invasive Magnetic Resonance Imaging (MRI) in conjunction with chemical shift imaging and spectroscopy to study variation in colony structures and *in vivo* mapping of oil domains in live naturally relevant sized colonies, such as large (700-1500 μm) and extra-large (1500-2500 μm) colonies. Furthermore, diffusion MRI has been exploited to get insight into the diffusion dynamics. The results show that MRI at high magnetic field (17.6 T) allows for mapping oil/hydrocarbon distribution in *B. braunii* directly in live colonies *in vivo*, thereby avoiding many of the elaborate and invasive analytical steps typically required to investigate algae. This study also provides for the first time non-invasive MRI means to obtain spatial information of oil distribution, and diffusion dynamics *in vivo*.

2.3 RESULTS

To cover the wide range of colony sizes exhibited by *B. braunii*, a dedicated Micro5 probe with a built-in strong gradient (2 T m^{-1}) was used. A 5mm volume RF coil with large vertical linear B1 range, which was specifically designed for a 17.6 T magnet, was selected to obtain sufficient resolution and signal to noise ratio. Fig. 2.1A shows a high-resolution morphological image of the colonies obtained by the Multi Slice Multi Echo (MSME) sequence. A detailed view of successive axial slices through the colonies is shown in supplementary figure 2.S1. The image slice in Fig. 2.1A shows multiple colonies with varying size but with highly similar colony structure. The colonies are ranging 700-1500 μm in diameter, and have moderately heterogeneous centres (black arrowhead). Henceforth I define these colonies as 'large colonies' to distinguish them from small colonies typically studied in literature (size 20-200 μm). The edges of these large colonies show an irregular surface area, with small extrusions representing botryoidal 'bunch-of-grapes' growth patterns. This is in line with morphological data observed for colonies of *B. braunii* with a combination of optical and electron microscopy (Tanoi, Kawachi and Watanabe, 2014). There is a dark band of 200-300 μm thickness near the surface of the colonies (black arrow). This band is most likely comprised of cells in the extracellular matrix, in which living cells occur predominantly near the surface. In this respect, recently Wijihastuti et al. (Wijihastuti *et al.*, 2016) have shown that when *B. braunii* is grown as biofilm, the living cells are confined to a surface layer of 20-60 μm .

Fig. 2.1 High resolution μMRI images of *B. braunii* colonies measured at a magnetic field of 17.6T. (A) Axial image of colonies ranging in size from 700-1500 μm . Images were obtained using the multislice multiecho (MSME) pulse sequence by averaging 4 echo images (average echo time, 13 ms; repetition time, 1500 ms; field of view (FOV), $5.0 \times 5.0 \text{ mm}$ and number of averages, 32) with a resolution of $19.5 \times 19.5 \times 100 \mu\text{m}^3$. Black arrowheads show central core and black arrows represent a clear dark band of 200-300 μm thickness surrounding the colonies. (B) Chemical Shift Selective Imaging (CSSI) of oil/hydrocarbon resonances. Imaging parameters were: repetition time, 1500 ms; echo time, 8.3 ms; number of averages, 64; resolution, $19.5 \times 19.5 \times 250^3 \mu\text{m}$. Receiver bandwidth used was 100 kHz. Excitation pulse of 600 Hz wide at -3.35 ppm with respect to water resonance was used. High oil containing region (region a, c) and low/no oil region (region b, d) can be clearly seen. (C) Matrix display of chemical shift imaging spectra. CSI data was recorded with a repetition time of 1100 ms, echo time of 12 ms and slice thickness was 0.25 mm. Total averages were 62. Resolution obtained was $156 \times 156 \times 250 \mu\text{m}^3$. Spectral width used was 10 kHz (13.33 ppm) and 32×32 matrix was reconstructed into 64×64 voxels. Inset: Representative spectra of single voxel showing residual water (1) and fat resonances (2). The main $-(\text{CH}_2)_n-$ signal in colonies is centred around 1.3 ppm, with side lobes from $-(\text{CH}_2)_n-\text{CH}_3$ up-field and $-\text{CH}_2-\text{CH}=\text{CH}-$, $-\text{CH}_2-\text{CH}_2-\text{COOR}$ extending downfield. (D) CSI voxel intensity thresholding. Signals between 0.80 to 1.25 ppm corresponding to fat were chosen to reconstruct CSI images and overlaid with corresponding T_2 -weighted MSME image using the Bruker CSI Visualisation Tool. Scale bar: 500 μm .



2.3.1 CHEMICAL SHIFT SELECTIVE IMAGING

The Chemical Shift Selective Imaging (CSSI) Sequence was utilised to resolve the spatial distribution of oil and water in *B. Braunii* large colonies (Fig. 2.1B and 2.S2 Fig.).

Though oils are generally present in very high proportion, other compounds such as algaenane share similar resonant frequencies as that of botryococcenes oils, causing them to be registered jointly in the CSSI images. In this work, I use the term oil to describe this hydrocarbon fraction, with it being understood that it is a part of a complex mixture of hydrocarbons and their derivatives. In the image slice shown in Fig. 2.1B, oil is localized in two defined concentric rings, that are the cross sections of an outer layer 100-150 μm thick (region a) and an inner layer 200-300 μm thick (region c). The outer layer can be

attributed to a hydrocarbon rich region of the extracellular matrix surrounding living cells [12]. The thicker inner layer shows a high signal intensity of oil that can be attributed to the hydrocarbon cross-linked network associated with cell remnants comprised of layers of mother cups (Wake, 1983). In addition, the thicknesses of both the hydrocarbon containing layers are uniform over the sample, across different colonies and over the range of colonies size (700-1500 μm) (Fig. 2.1B). The region of 100 μm thick (region b) between the two oil layers corresponds to a transition region between the living cells (layer a) and the cell remnants (layer c). Interestingly, in contrast to the outer layers, oil was not detected in the centres of the colonies. It is unlikely that oil is present in this region and CSSI sequence could not detect it due to line broadening that may arise due to presence of paramagnetic ions, since the MSME imaging works well in these areas (Fig. 2.1A).

The contrast between oil rich and oil poor regions is greater for large colonies compared to smaller colonies (region d) (Fig. 2.1B). Localisation of the water signal by CSSI reveals the presence of high concentrations of water in the centre, but not in the outer layers of the colony (2.52 Fig.). In addition, in many colonies the distribution of water in the centre region is inhomogeneous and gradually varies, *i.e.* without distinct boundaries (2.52 Fig.).

2.3.2 CHEMICAL SHIFT IMAGING

The content and distribution of oil were further analysed spectroscopically by chemical shift imaging (CSI). Chemical shift imaging exploits differences in the local magnetic field experienced by protons to capture localized spectra in a 2D or 3D matrix. Because the resonance frequency of protons depends on the local magnetic field seen by the proton, protons in lipid molecules resonate at different frequency compared to those in water molecules. CSI utilises this principle of chemical shift for localised spectroscopic imaging, by forgoing the readout gradient used in imaging (including CSSI) and instead incorporating an additional phase encoding step. The pulse sequence then captures a full spectrum for each encoded voxel. CSI therefore allows mapping the spatial distribution of hydrogen nuclei associated with water or with lipid molecules. CSI has an advantage over single- or multiple-voxel localization techniques since there is no chemical shift artefact problem in CSI (Brown, 1992). Because of this, it is useful for high-field *in vivo* MRS applications in which the chemical shift dispersion increases linearly as a function of B_0 . CSI data are presented as a matrix array of spectra in Fig. 2.1C. In a representative spectrum, taken from the highlighted area in Fig. 2.1C, two major peaks are visible belonging to water (1) and oil (2) within colonies (inset Fig. 2.1C). The main $-(\text{CH}_2)_n-$ signal of lipid in colonies is centred around 1.3 ppm, with side lobes from $-(\text{CH}_2)_n-\text{CH}_3$ up-field and $-\text{CH}_2-\text{CH}=\text{CH}-$, $-\text{CH}_2-\text{CH}_2-\text{COOR}$ extending downfield. A map of oil or water can be displayed in colour and overlaid on an MR image of the same slice. The CSI image of oil signal integration is depicted in Fig. 2.1D, revealing that indeed all large colonies (700-1500 μm) exhibited a double oil layer (a, c) and very low oil at the centre of the colony (d). The inner oil ring appears to be higher in oil concentration as compared to the outer oil ring as seen in both CSSI and CSI measurements (Fig. 2.1B and 2.1C, D).

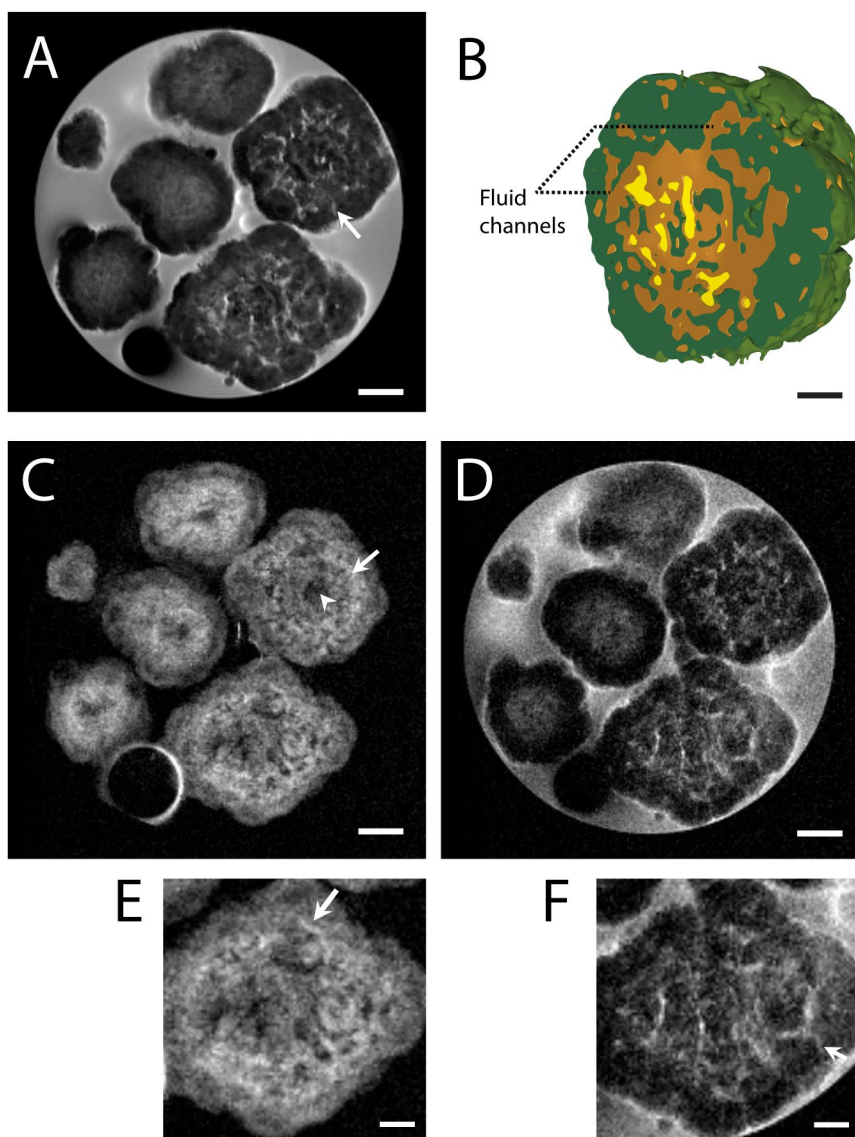


Fig. 2.2 Diversity in colony types visualised at high magnetic field (17.6T). (A) Image was obtained using 2D MSME pulse sequence (echo time, 13 ms; repetition time, 1500 ms; number of averages, 128) with an in-plane resolution of $19.5 \times 19.5 \mu\text{m}$. Large and extra-large size colony types can be discerned. Arrow indicates a fluid channel. (B) 3D-volume reconstruction of extra-large colony based on voxel intensity thresholding, showing presence of fluid channels. (C) Spin echo CSSI of oil/lipids showing a pattern of low (arrowhead) and high (arrow) oil containing regions. Oil appears absent in the centre of the colony. (D) Spin echo CSSI of water signal. Water channels are visible in extra-large colonies. (E) Enlarged area of (C) showing possible septum (white arrow). (F) Enlarged area of (D) showing fluid channels, some of them reaching the colony surface (white arrowhead). Scale bar: $500 \mu\text{m}$ (A,C,D) and $250 \mu\text{m}$ (B,E,F).

Thus, CSI is not only able to accurately measure protons of lipid/oil but also assessed its distribution and relative intensity in localized domains in *B. braunii* colonies.

2.3.3 EXTRA-LARGE COLONIES SHOW A SIGNIFICANTLY HETEROGENEOUS STRUCTURE

Within samples of *B. braunii* cultures, some extra-large colonies are also visible (ranging between 1500-2500 μm diameter) (Fig. 2.2A). These colonies show a significantly heterogeneous structure especially at their centres, as compared to 'large sized' colonies (<1500 μm). Henceforth I refer to these colonies as 'extra-large colonies'. Interestingly, fluid channels reaching the surface are observed in these extra-large colonies, and are indicated with a white arrow in Fig. 2.2A. The 3D intensity reconstruction made from one of the extra-large colonies shown in Fig. 2.2B reveals heterogeneity in the colony and the presence of channels reaching the surface.

Analysis of the oil distribution in extra-large colonies revealed that oil was spread throughout colonies, but with large local variations (Fig. 2.2C). The double ring structure as observed for smaller colonies, was also present in extra-large colonies, but the hypo-intense region between the rings was less clear. In contrast to smaller colonies, the central part of the extra-large colonies contains a pattern of low (arrowhead) and high (arrow) concentrations of oil. Conversely, water distribution for the extra-large colonies shown in Fig. 2.2D indicates that water is distributed throughout the colony in the form of channels, with some of these channels or interfaces reaching the colony surface (Fig. 2.2F). Curiously, some hyper intensities were observed in the oil CSSI image, which can be speculated to be a part of the interface or septum between sub-colonies (Fig. 2.2E, arrow). A false colour overlay image generated from Fig. 2.2C and 2.2D revealed distribution of the water and oil signal (2.53 Fig.). In general, local highs in oil are correlated with a local depletion of water and vice versa. Additionally, the area in between the oil rings of a large colony appears dark in 2.53 Fig., implying that both oil and water signals are weak in this area (white arrow).

2.3.4 T_1 AND T_2 RELAXATION PROPERTIES OF *B. BRAUNII* COLONIES

Morphological variation and differences in oil concentrations between colonies can also be reflected in proton longitudinal (T_1) and transverse (T_2) relaxation properties, which can be used as surrogate biomarkers for a colony type. In order to evaluate relaxation variations within colonies, several representative regions of interest (ROI) were selected within a large sized (top left) and in an extra-large colony (middle) (Fig. 2.3A). The T_1 and T_2 relaxation times were calculated and compared with observed structural details shown in tabular form in Fig. 2.3D.

The longest T_1 was observed at the inner centre of the large sized colony, (region 1, ~1100 ms), while the outer central region shows significantly shorter T_1 (regions 2-4, ~753 ms). Hence, it can be concluded that the composition of the central water-containing region of these colonies is heterogeneous. The outer layers of these colonies

(regions 5-7) exhibit shorter T_1 (average ~ 578 ms). The difference in T_1 for the central and surrounding regions is also clearly visible in the T_1 map depicted in Fig. 2.3B, showing a long T_1 for a confined light blue area in the centre of the large sized colony (white arrow) and significantly shorter relaxation times for the surrounding regions (dark blue, white arrowhead).

Extra-large colonies exhibited a variation in the distribution of T_1 relaxation time. The T_1 varied between ~ 560 ms to ~ 704 ms (regions 8-10) within the central part of the colony. The heterogeneity of T_1 in the central part is more pronounced in extra-large colonies as compared to large colonies. The colony outside ring T_1 was very similar in comparison to large colonies, ranging from ~ 539 to ~ 628 ms (regions 11-13).

Fluid channels within the extra-large colony have varied T_1 relaxation times as denoted by asterisks in Fig. 3A (~ 591 ms and ~ 716 ms). Based on these values, it can be concluded that there is possibly variation in fluid composition corroborating the findings from CSSI imaging. However, visibility of fluid channels depends on both T_1 and T_2 contrast, since the image intensity is not directly correlated with the T_1 relaxation time.

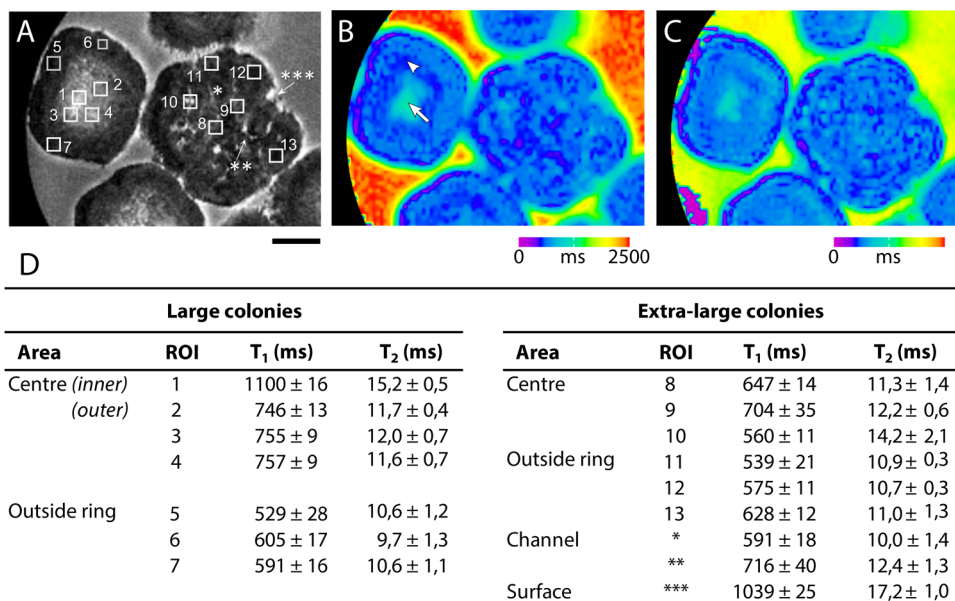


Fig. 2.3 T_1 and T_2 relaxometry and mapping of *B. braunii* colonies. Relaxation measurement was performed using RAREVTR sequence (TR-array, 5500-200 ms; TE, 27-4.5 ms; number of averages, 16; matrix size, 128×128 ; FOV, 0.5×0.5 mm; resolution was $39 \times 39 \times 250 \mu\text{m}^3$). (A) A representative image showing regions of interest (ROI) placed on two representative colonies (one large and one extra-large size colony) for calculating T_1 and T_2 relaxation times. Scale bar: 500 μm . (B) T_1 Map derived from RAREVTR sequence, showing the region of high (white arrow) and low T_1 (white arrowhead). Colour scale was generated with Paravision 'colour 256' scheme which ranges from 0 to 2500 ms. (C) T_2 map derived from RAREVTR sequence showing a sharp edge of low T_2 surrounding all colonies (black arrow). Colour scale ranges from 0 to 40 ms. (D) Tabulated data of T_1 and T_2 relaxation times calculated from ROI indicated in (A).

The transverse relaxation time or spin-spin relaxation time, T_2 , is a specific attribute of spins, which depends on their surroundings. Interaction between spins, for example, coupling to neighbouring nuclei, destroys the phase coherence; therefore, the T_2 relaxation time can be a sensitive indicator of the variation in the microenvironment within a colony volume. The centre of the large sized colony exhibited the longer T_2 (15.2 ± 0.5 ms) as seen by the blue-green region in Fig. 2.3C. In contrast, the outer central regions (region 2-4) have significantly lower T_2 (~ 11.6 – 12.0 ms). The edge of the colony show lower T_2 relaxation time (ranging from ~ 9.7 ms to ~ 10.6 ms (region 5-7), which is also reflected by a dark purple ring seen in the T_2 map (Fig. 2.3C, black arrow).

In extra-large colonies, the T_2 times measured in the centre show high variation (ranged from ~ 11.3 ms to ~ 14.2 ms). In contrast, in large size colonies, T_2 relaxation time spread was smaller in the centre (region 2-4), confirming that the sample composition may be more homogeneous in these colonies. On the other hand, the T_2 relaxation times in the centre of the extra-large colonies was significantly different, especially due to the presence of fluid channels (* and **). Overall, these results confirm the imaging data that extra-large colonies are more heterogeneous in appearance as compared to normally sized colonies. In general the variation in T_2 over different region is rather small, which could be due to the fact that T_2 values of different fraction (a mixture of water and hydrocarbon) within a voxel are averaged. In addition, high level of hydrocarbons do not appear to translate to longer T_2 relaxation times. This may possibly be a result of enhanced rate of relaxation of hydrocarbons due to interactions with the local environment.

A hyper-intense ring is visible at the surface of all the colonies as seen clearly in Fig. 2.3A. This ring was especially visible under $TR \leq 1500$ ms. This hyper-intense ring is also reflected in the T_1 map (Fig. 2.3B) and to a lesser extent present in the T_2 map (Fig. 2.3C). In the T_1 map, the ring appears as a transition zone with increasingly higher T_1 further away from the colony. The transition zone (***) contained a long T_1 of ~ 1039 ms. It is likely caused by diffusion mediated interactions between the surrounding medium and the exopolysaccharide fibrillary sheath of *B. braunii* (Díaz Bayona and Garcés, 2014; Furuhashi *et al.*, 2016).

2.3.5 DIFFUSION BEHAVIOUR IS CORRELATED TO COLONY SIZE

To resolve correlations between colony structure and diffusion dynamics in water and oil rich regions, apparent diffusion coefficients (ADC) in colonies were assessed with a series of images captured with incrementally increasing diffusion sensitising gradient strengths (Fig. 2.4). Diffusion weighting works on the basis of twin dephasing gradient echoes with opposing direction. Because of this, only stationary protons are rephased completely. Protons that diffuse experience a net gradient and are dephased proportional to the distance travelled. In general, free water is most strongly dephased leading to a progressive darkening of background medium signal. Under a B-value of 1182 s mm^{-2} , the water signal is already strongly diminished (Fig. 2.4A).

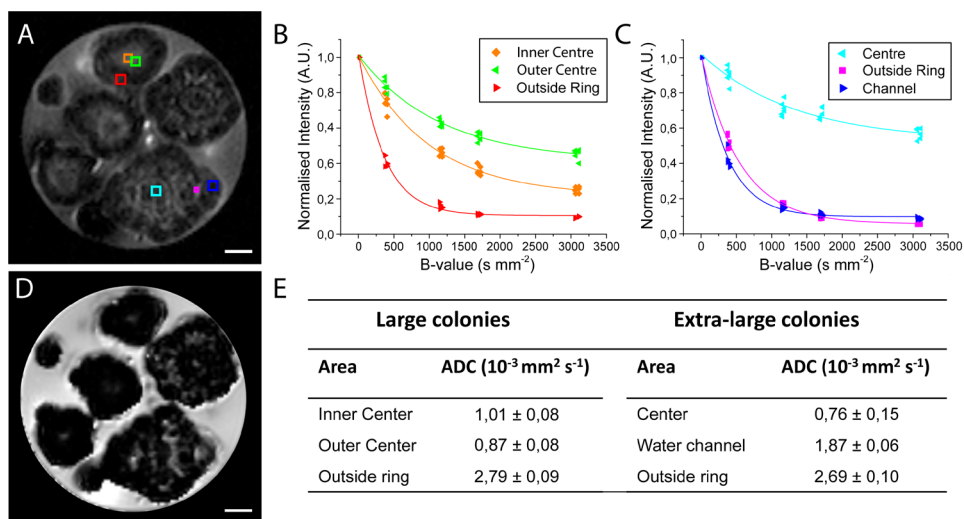


Fig. 2.4 Diffusion is strongly correlated to colony size. Diffusion measurements were performed using a spin echo pulse sequence containing a pair of mono-polar diffusion-sensitising gradients (TR, 1500 ms; TE, 10.1ms; 16 averages; diffusion gradient duration 2.5 ms and gradient separation of 5 ms; effective B-values range: 14, 406, 1182, 1723, 3123 s mm^{-2}). (A) A diffusion image showing regions of interest (ROI) placed on two representative colonies (one large and one extra-large sized colony) for calculating apparent diffusion coefficient (ADC). Colour-coded ROIs are correlated to the signal decay curves in (B) and (C). Diffusion intensity decay curves normalized to unity for representative regions of interest on large sized (B) and extra-large size (C) colony. (D) ADC map generated through Bruker internal 'dtraceb' algorithm. (E) Table of calculated ADC values for regions of interest shown in (A). Scale bar in A and D: 500 μm .

Remarkably, oil-rich regions in large colonies exhibited very low dephasing under strong gradients, as indicated by bright signal in these areas (2.54 Fig. and 2.51 Video). It implies that diffusion is highly restricted in oil rich regions, which appears to hold to a lesser extent for extra-large heterogeneous colonies as well.

Quantification of diffusion was achieved by calculation of apparent diffusion coefficients (ADC) for selected ROIs placed on two different types of colonies as shown in Fig. 2.4A. A mono-exponential model for fitting shown in Fig. 2.4B and Fig. 2.4C reflects the combined contributions of the water fraction and hydrocarbon fraction to the ADC. Because the hydrocarbon fraction is resistant to signal attenuation at the diffusion sensitising gradient strengths used (3123 s mm^{-2}), it can be assumed to be constant for the purposes of mono-exponential curve fitting. Thus ADC reflects predominantly water diffusion. ADCs were also calculated at each voxel position for display in the ADC map depicted in Fig. 2.4D. As summarized in tabular form in Fig. 2.4E, the centre of a large size colony exhibited some restriction of diffusion $1.01 \times 10^{-3} \text{ mm}^2 \text{ s}^{-1}$ as compared to outside medium ($2.4 \times 10^{-3} \text{ mm}^2 \text{ s}^{-1}$). The immediate area outside the central core (outer centre) was on average lower in diffusion at $0.87 \times 10^{-3} \text{ mm}^2 \text{ s}^{-1}$ corresponding to the thickest oil band observed in Fig. 2.2C. The diffusion was found to be highest at the edge of colony ($2.79 \times 10^{-3} \text{ mm}^2 \text{ s}^{-1}$). This value was even higher than the values observed for the

surrounding medium. This could possibly be due to enhanced relaxation rates related to the 'halo' phenomenon observed in T_1 and T_2 maps. Most anisotropy in diffusion was observed for the centre of extra-large colonies (Fig. 2.4), while the region containing a fluid-filled interface was comparatively low in anisotropy.

Within the centres of extra-large heterogeneous colonies, a water channel exhibited an ADC value of $1.87 \times 10^{-3} \text{ mm}^2 \text{ s}^{-1}$, interestingly higher than the large colony centre. Other parts of the interior exhibited low apparent diffusion coefficient ($0.76 \times 10^{-3} \text{ mm}^2 \text{ s}^{-1}$). Similar to the large sized colony, edge ADC of extra-large colonies is very high ($2.69 \times 10^{-3} \text{ mm}^2 \text{ s}^{-1}$).

The ADCs are highly co-localised with the T_1 relaxation characteristics shown in Fig. 2.3B. The presence of interfaces between sub-colonies provides clues for possible anisotropy in diffusion, therefore six equally spaced diffusion directions were recorded, which also served to avoid background interference from imaging gradients in ADC calculation.

2.4 DISCUSSION

This work provides the first non-invasive MRI means to analyse morphology and internal structural variation of *B. braunii* colonies and obtain spatial information of oil distribution, and diffusion dynamics *in vivo*. Large size colonies (700-1500 μm diameter) show a well-defined central core and number of surrounding layers. Previous studies indicate that the central core may be filled with the remainder of dead cells and their extracellular matrix (Guy-Ohlson, 1998). If this is the case, it would stand to reason that oil would also be present here. However, this does not appear to be the case as illustrated by the absence of oil in colony centres measured by CSSI of oil resonances (Fig. 2.1B). Though oil has been widely proposed as having important energy storage functions for algae, it has been reported that *B. braunii* is not capable of degrading its own long-chain hydrocarbons (Schenk *et al.*, 2008)(Largeau, Casadevall and Berkloff, 1980). Thus, it is likely that algae do not store oil reserves in the centre, and oil found in the colonies is restricted either to the areas where living cells are localized and producing the oil or in the area immediate next to the living cell layer containing extracellular matrix. It is known that in large *B. braunii* colonies, living cells are exclusively located on outer rings of the colony (Wake, 1983). This is in contrast to small *showa* colonies (50-200 μm), where live cells are distributed in subcolonies throughout the entire colony (Suzuki *et al.*, 2013). The CSSI, CSI as well as relaxation data clearly show that these outer ring areas of the colony contain high concentration of oil. The two concentric oil layers may be attributed to the complex extracellular matrix connecting individual cells (Weiss *et al.*, 2012; Uno *et al.*, 2015). This notion is in line with the low apparent diffusion coefficient observed on these oil containing rings (Fig. 2.4).

In contrast to large colonies, the extra-large *B. braunii* colonies (1500-2500 μm diameter) show a significantly different internal makeup, namely an altogether more heterogeneous central part and less defined outer rings. These colonies correspondingly contain a more heterogeneous distribution of oil including at the central part of the

colonies. The CSSI imaging of oil shows that less defined outer ring of these extra-large colonies is rich in oil content. The finding that oil is localised near the surface of both large colonies and extra-large colonies may have some implications for oil extraction from *B. braunii* colonies. For example, solvent-based extraction of oils for larger colonies could occur at similar efficiencies compared to smaller colonies, since the required penetration depth stays relatively equal (Fig. 2.1B) (Wijihastuti *et al.*, 2016).

One of the most important findings of this study is the observation of the existence of fluid channels, which could be visualized for the first time in live *B. braunii* colonies. In addition, by exploiting various MRI methods it was possible to characterise the composition of these channels. However, the role of fluid channels in extra-large colonies is not clear. Since the network of water based fluid channels was found to be reaching to the surface of the colonies, its role in nutrient diffusion could be possible. The apparent diffusion coefficient measurements show that fluid channels have relatively low diffusion restriction as compared to other areas of the colonies.

The results of MSME imaging, relaxation and diffusion measurements also provide a clue toward nutrient and solvent exchange at the surface of colonies. The surface ring hyperintensity found in MSME imaging, is attributed to diffusion generated T_1 contrast as it appears most strongly under short TR (≤ 1500 ms) (Brownstein and Tarr, 1979; Kaufmann *et al.*, 2008). Hyperintense rings are known to arise from porous organic surfaces in contact with bulk water (Kaufmann *et al.*, 2008). Although the thickness of the colony fibrillary sheath is known to be circa 4-7 μm , the resulting ring phenomenon is much larger, in the order of 50 μm (Fig. 2.3) (Uno *et al.*, 2015). It can be postulated that this reflects a highly porous nature of the colony sheath to small molecules including water. Diffusion coefficient was found to be very high at the edge of the colony. This value was even higher than observed for surrounding medium. This could possibly be due to enhanced relaxation rates linked to the 'halo' phenomenon observed in T_1 and T_2 maps (Fig. 2.3 and 2.4).

The extra-large heterogeneous colonies observed in this work may have been formed by the combination of several smaller colonies or by having multiple seed-colonies. Important to this argument is the knowledge that *B. braunii* is known to form biofilms as well as inclusion in bacterial biofilms (Rivas, Vargas and Riquelme, 2010; Zhang and Lin, 2014). This property is especially useful in Biofilm Photobioreactors (PBRs), which are seen as a promising avenue for commercialisation (Hoh, Watson and Kan, 2016). Linking of sub colonies together to form an extra-large colony is thus certainly plausible; possible in the life-cycle of *B. braunii*, and would provide a fast biomass accumulation scenario. Importantly, it provides a plausible mode of water channel formation, arising from interstitial space between growth clusters of *B. braunii*. These channels are particularly well visualised when viewed as a maximum intensity projection MIP, as seen in supplementary video (2.S2 Video). Curiously, segmentation of cells by retaining-wall shells is well known, but these shells are rich in hydrocarbons, not water (Weiss *et al.*, 2012). Thus, my hypothesis may mean the organisational model for extra-large colonies

is more complex than the current life cycle models of smaller colonies in literature. My findings on colony organization are summarised graphically in 2.55 Fig. It can be concluded that imaging secondary metabolites directly and *in vivo* using MRI is feasible and provides an advantageous platform for the study of oleaginous algae and biofilms.

2.5 MATERIALS AND METHODS

2.5.1 BOTRYOCOCCUS BRAUNII CULTIVATION

B. braunii, variant *showa*, was grown in a bubble column on a modified CHU-13 medium (Chu, 1942). The medium was composed of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (108 mg L^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (200 mg L^{-1}) and K_2HPO_4 (104.8 mg L^{-1}), KNO_3 (1.2 g L^{-1}), $\text{Na}_2\text{O}_4\text{Se}$ (9.44 mg L^{-1}), FeNaEDTA (20 mg L^{-1}). Micronutrients traces consisted of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.08 mg L^{-1}), $\text{ZnSO}_4 \cdot 5\text{H}_2\text{O}$ (0.19 mg L^{-1}), $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ (0.09 mg L^{-1}), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (1.27 mg L^{-1}), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.06 mg L^{-1}), H_3BO_3 (2.86 mg L^{-1}) and concentrated H_2SO_4 (0.01 ml L^{-1}). KNO_3 concentration was chosen so as to minimise the potential of nitrogen growth limitations. Citric acid was omitted from the medium composition, ensuring phototrophic growth due to the absence of a carbon source. All ingredients were autoclaved separately and final medium was adjusted to pH 7.2 with NaOH. Cultures were transferred to fresh medium every 2 weeks to maintain exponential growth.

Culture illumination was provided by continuous cool LED lighting with a Correlated Colour Temperature (CCT) of 4300K and light intensity of 30 Klux or approximately $450 \mu\text{mol s}^{-1} \text{ m}^{-2}$. Temperature was maintained at $25 \pm 1^\circ \text{C}$ under continuous sparging with ambient air. To prevent evaporation due to gas bubbling, air was wetted by sparging through distilled water prior to entering the culture vessel. Cultures were grown in high light ($450 \mu\text{mol s}^{-1} \text{ m}^{-2}$) under continuous illumination for 15 weeks. Under these conditions colonies achieved remarkably large and extra-large sizes ($700\text{--}2500 \mu\text{m}$). For the MRI measurements, the colonies were transferred using a 2 ml volume pipet to a glass dish, drained of excess medium and then transferred by spatula to a 5 mm NMR tubes. Teflon stoppers were inserted into the tubes to prevent displacement and dehydration of the colonies. Colonies were kept in culture medium during *in vivo* MRI measurements.

2.5.2 MRI ACQUISITION

All experiments were performed on a 17.6 T (750 MHz), vertical 89 mm wide bore magnet (Bruker Biospin, Ettlingen, Germany) in combination with an Avance I console. A Bruker Micro5 probe with 5mm birdcage resonator and a built-in $48 \text{ mT m}^{-1} \text{ A}^{-1}$ (1.92 T m^{-1} at 40 A) gradient system coupled to BAFPA 40A amplifiers was used for all measurements. All spectrometer operation was controlled by a Linux PC running Topspin 2.0PV and Paravision 5.1. Sample temperature was maintained at $293 \pm 1 \text{ K}$ through gradient water-cooling.

2.5.2.1 MULTI SLICE MULTI ECHO

Anatomical reference images were acquired using a Multiple Slice Multi Echo Sequence (MSME), refocusing magnetisation from an initial 90° pulse with a 180° pulse echo train.

The following basic parameters were used for Fig. 1A: echo train length = 4; All echo images were summed to produce a composite image. Average echo time (TE) = 13ms; Repetition time (TR) = 1500 ms; receiver bandwidth = 100 kHz; number of averages = 32; Field of View (FOV) = 5 × 5 mm²; matrix size = 256 × 256; and resolution = 19.5 × 19.5 × 100 μm³.

2.5.2.2 RAREVTR T_1 AND T_2 MAPPING

Longitudinal (T_1) and transverse (T_2) relaxation maps were obtained using a Rapid Acquisition with Relaxation Enhancement with Variable TR (RAREVTR) pulse sequence. The sequence used a saturation scheme (*i.e.*, varied TR) to acquire T_1 and used a multi-echo CPMG scheme (*i.e.*, varied TE) to acquire T_2 . The following parameters were used: TR-array = 200, 400, 800, 1500, 3000, 5500 ms; TE = 4.5, 9.0, 13.5, 18.0, 22.5, 27 ms for each TR; echo spacing = 4.5 ms; RARE factor = 1; Number of averages (NA) = 16; matrix = 128 × 128; acquisition time = 4 hours 52 minutes. The field of view was 5 × 5 mm² with a thin slice of 0.25 mm thick to prevent partial volume effects, resulting in a resolution of 39 × 39 × 250 μm³. The voxel volume was 3.8 × 10⁻⁴ mm³ and a receiver bandwidth of 100 kHz was used. A single slice was acquired to prevent interslice modulation effects. ROIs were manually defined using an Image Sequence Analysis tool package (ISA) (Paravision 5.1, Bruker), Transverse relaxation was calculated using the fit function: $M(t) = C \left(\frac{t}{T_2} \right)$, where C = signal intensity, and T_2 = transverse relaxation time. The T_1 values were determined by image sequence analysis using a fit function: $M(t) = M_0 \times (1 - e^{\left(\frac{t}{T_1} \right)})$, where M_0 is the equilibrium magnetization. Built-in functions were used to generate T_1 and T_2 relaxation maps from the parameter fitting on a pixel by pixel basis.

2.5.2.3 CHEMICAL SHIFT SELECTIVE IMAGING

Chemical Shift Selective Imaging (CSSI) was used to acquire selective water and fat/oil images separated on the basis of biochemical composition, as opposed to differences in T_2 in inversion-recovery methods (Haase *et al.*, 1985). A narrow bandwidth 90° Gaussian pulse was used for on resonance frequency selective excitation. FOV and Matrix were identical to MSME anatomical imaging: FOV = 5 × 5 mm², Matrix = 256 × 256. Further basic parameters are as following: Receiver bandwidth = 100 kHz, TR = 1500 ms. Measurements were averaged 64 times for a total acquisition time of 6 hours 49 minutes. Two separate measurements were performed with 1500 Hz and 600 Hz of excitation bandwidth respectively. The water oil frequency difference was determined to be 2.7 kHz at 17.6 T. Signal cross contamination was prevented by choosing a bandwidth of 1.5 kHz, covering the whole oil containing spectral region.

For Fig. 1B and 1C, Excitation bandwidth = 1500 Hz, -3.00 ppm. Slice thickness = 500 μm; resolution 19.5 × 19.5 × 500 μm³ and TE = 6.2 ms. Number of averages of Fig. 4B = 220. For Fig. 2B and 2C, Excitation bandwidth = 600 Hz, oil excitation was offset to 1.75 ppm. Slice thickness = 250 μm; resolution = 19.5 × 19.5 × 250 μm³ and TE = 8.3 ms.

2.5.2.4 CHEMICAL SHIFT IMAGING

Chemical Shift Imaging (CSI) was utilised in spin echo slab selective mode. CSI employs two orthogonal phase encoding steps with pulsed gradients to record a pure spectroscopic echo upon acquisition, instead of a conventional readout gradient used in imaging. A Hanning function weighted k -space acquisition scheme was utilised, as implemented by the Bruker '*weighted*' measuring method, for an improved Spatial Response Function (SRF). Basic parameters were as follows: TR = 1100 ms; TE = 12 ms; Matrix = 32×32 ; FOV = $5 \times 5 \text{ mm}^2$; slice thickness = 250 μm ; resolution = $156 \times 156 \times 250 \mu\text{m}^3$; number of scans = 45,000. Data was reconstructed into a 64×64 matrix with linear smoothing for display. Excitation and refocusing was achieved using Sinc3 pulses with a bandwidth of 8000 Hz. Echoes were captured into 2048 points in 204.80 ms, spectral resolution = 2.4 Hz per point; and Spectral width = 10 kHz (13.3 ppm). Magnetic field homogeneity in the selected volume was optimized by shimming the water resonance. A VAPOR suppression scheme of 625 ms was applied for efficient water signal saturation. Interpulse radiofrequency delay was 150, 80, 160, 80, 100, 37.2, 15 ms between seven hermite shaped CSSI modules. RF bandwidth = 900 Hz, excitation offset = -75 Hz (-0.1 ppm).

2.5.3 DIFFUSION WEIGHTED MRI

Diffusion measurements were carried out with a spin echo pulse sequence containing a pair of mono-polar diffusion-sensitising gradients. Gradient orientations were isotopically distributed in six directions. Gradient strength ranged from 14, 406, 1182, 1723, 3123 s mm^{-2} effective B-values, including imaging gradients. Diffusion gradient duration (δ) of 2.5 ms was combined with 5 ms diffusion gradient separation (Δ), for a total TR and TE of 1500 and 10.1 ms respectively. To obtain sufficient SNR 16 averages were recorded resulting in a total acquisition time of 16 hours. FOV was $5 \times 5 \text{ mm}^2$, matrix size 96×96 and slice thickness of 0.5 mm, resulting in a resolution of $52 \times 52 \times 500 \mu\text{m}^3$. Receiver BW was 100 kHz.

2.5.4 POST-PROCESSING AND ANALYSIS

All experimental data were acquired and processed using Paravision 5.1 (Bruker Biospin, Ettlingen, Germany) running on CentOS 3 and figures were prepared in Adobe Photoshop CC 2015.3 and Adobe Illustrator CC 2015.2 (Adobe Systems Incorporated, Mountain View, California, USA). A false colour image (S3 Fig.) was generated by overlaying CSSI Oil signal on CSSI Water signal using the '*lighten*' transfer mode.

2.5.4.1 3D VOLUME RECONSTRUCTION MODELS

3D-MSME data were exported to DICOM using Paravision 5.1, consequently reconstructed in Slicer 3D 4.8 (www.slicer.org, [40]) and further processed in Meshmixer (Autodesk, San Rafael, California, USA).

2.5.4.2 PROCESSING OF CSI DATA

Integration of selected signal areas in magnitude mode were overlaid on MSME reference spectra using the Bruker CSI Visualisation Tool. A representative 1D spectrum was processed and exported using CSI-Tool (Bruker).

2.5.4.3 PROCESSING OF DIFFUSION DATA

Diffusion data was processed with a trapezoid windowing function to remove DC offset artefact (window maximum between 12.5% and 87.5% of acquisition window). Windowed results were consequently analysed using the Bruker Image Sequence Analysis Tool. Signal intensity and standard deviation were derived from the internal fitting function 'dtraceb': $S(b) = A + Ie^{Db}$ Where A is an Offset and I the amplitude of diffusion with diffusion coefficient D . Several Regions of Interest (ROI) were selected and used for further calculations. Tabulated data containing ROI decay curves was exported for further analysis. Because the system contains two fraction, *i.e.*, water (w) and hydrocarbons (h), normally bi-exponential fitting is required to accurately describe the system: $S(b) = f_w e^{-bD_w} + f_h e^{-bD_h}$ Where $f_h = 1 - f_w$. However, because the maximum gradient strength used was only 3123 s mm^{-2} , oil is not significantly attenuated because: $b \ll \frac{1}{D_h}$. This means that the diffusion attenuation for the oil/hydrocarbon fraction simplifies to: $e^{-bD_h} \approx 1$. As a consequence the system can then be described mono-exponentially as follows: $S(b) = f_w e^{-bD_w} + f_h$. The mono exponential fitting was performed in OriginPro 9.1.0 with Levenberg-Marquardt algorithm iteration (OriginLab Corporation, Northampton, Massachusetts, USA).

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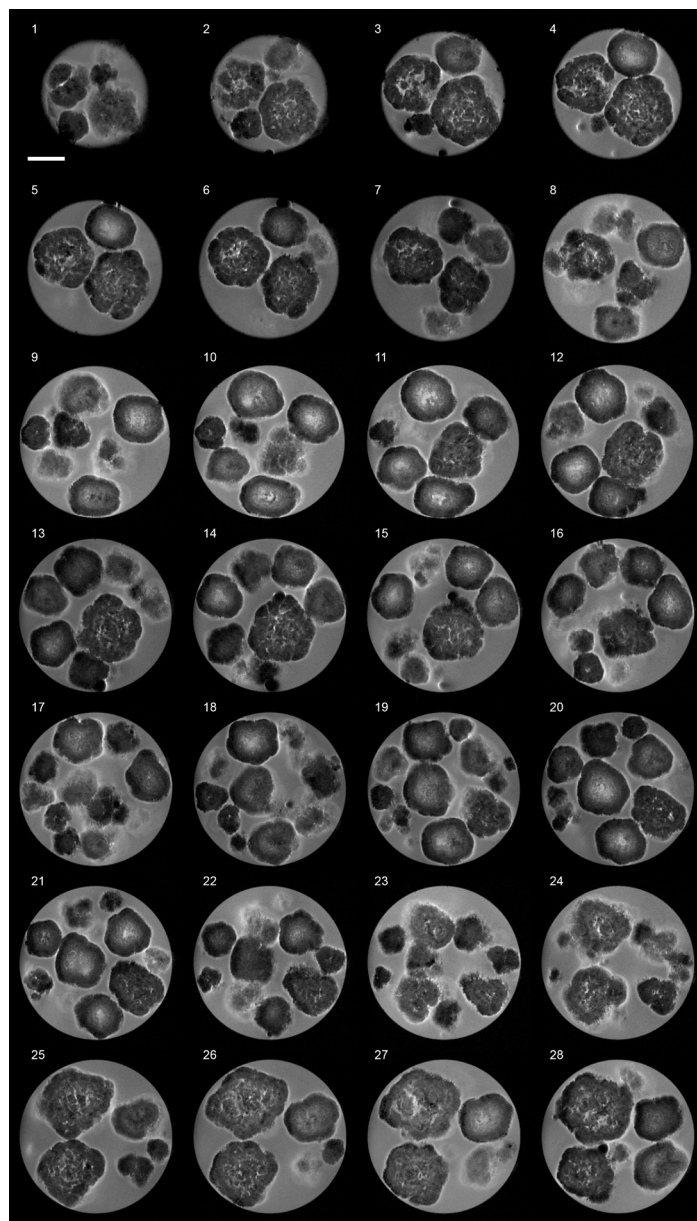
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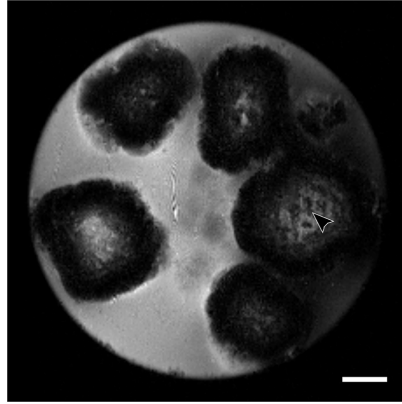
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Zhang, Y. S. H. and Lin, X. X. X. (2014) 'Biofilm formation and lipid accumulation of attached culture of *Botryococcus braunii*'. doi: 10.1007/s00449-014-1287-1.

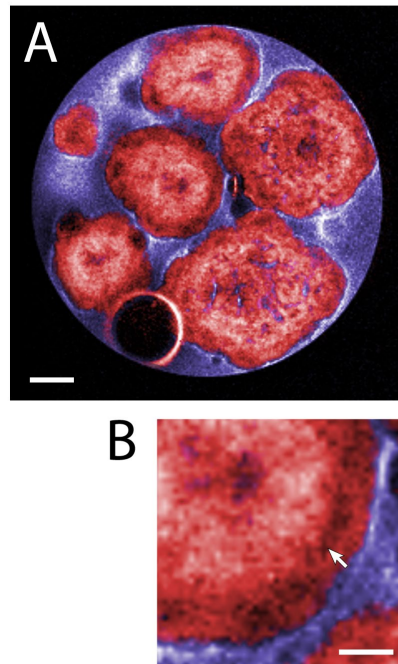
2.8 SUPPORTING INFORMATION



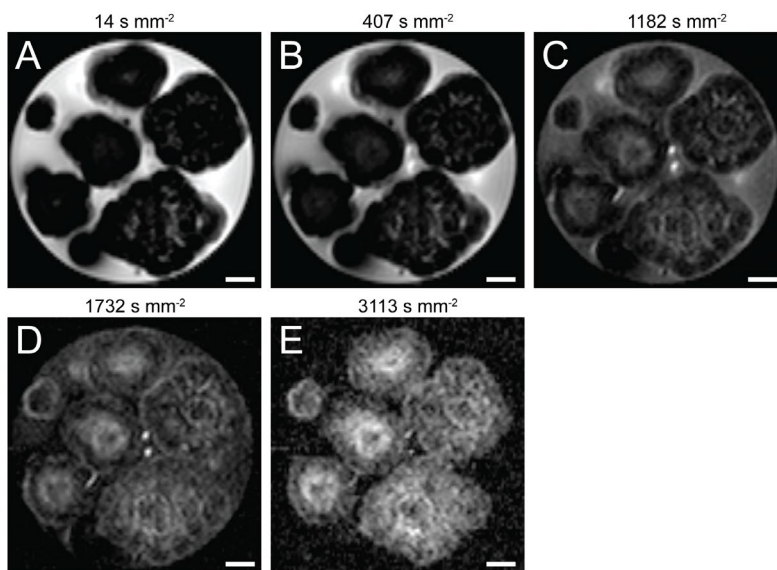
2.S1 Fig. Pseudo-3D MSME successive axial slices through the colonies showing variation in size and structure of *B. braunii* colonies. Imaging parameter used (TR, 1500 ms; TE, 13 ms; acquisition time, 3 h 24 m; Average, 32). Resolution $19.5 \times 19.5 \times 200 \mu\text{m}^3$ captured with a matrix of 256×256 . Scale bar: 1000 μm .



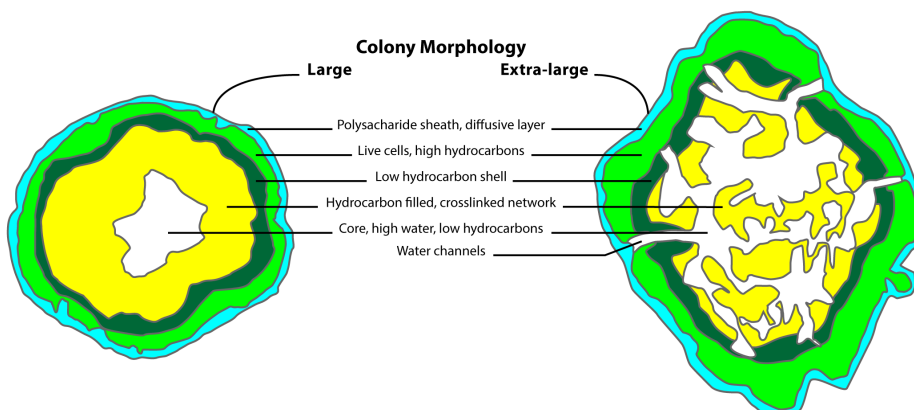
2.52 Fig. CSSI of water resonances. Imaging parameters used are: repetition time, 1500 ms; echo time, 8.32 ms; number of averages, 64 and resolution, $19.5 \times 19.5 \times 250 \mu\text{m}^3$. Receiver bandwidth used was 100 kHz. Excitation pulse of 600 Hz wide at water resonance. Inhomogeneity in the distribution of water in the centre is seen (arrowhead). The water signal was found to be low in two oil containing bands (arrows). Scale bar: 500 μm .



2.53 Fig. False colour overlay of oil/lipid signal in red and water signal in blue. An enlarged area of extra-large colony in (A) is shown in (B) depicting a possible septum (white arrow). Scale bar 500 μm (A), 100 μm (B).



2.54 Fig. Diffusion Weighted Images captured for ADC Mapping. Individual images from the +Z diffusion weighting direction. TR, 1500 ms; TE, 10.1 ms; 16 averages; diffusion gradient duration 2.5 ms and gradient separation of 5 ms; effective B-values range: 14, 406, 1182, 1723, 3123 s mm⁻². Scale bar 500 μ m.



2.55 Fig. Summarising model of *B. braunii* colony organisation. Shown are a large colony (700-1500 μ m) (left) and extra-large colony (>1500 μ m) (right). Boundary model derived from integration of MSME and CSSI imaging results on morphology of the colonies seen in Fig. 2.

Note: to view video material, please visit the online version of this thesis at the Leiden University Repository.

2.S1 Video Animated movie of Diffusion weighting steps. Movie version generated from S4 Fig. Video length 6s, 251 frames. Scale bar 500 μm .

2.S2 Video Maximum Intensity projection of 3D MSME imaging. Maximum Intensity Projection Video (MIP) of 3D-MSME data of multiple colonies submerged in perfluorodecalin (PFD), which highlights the difference in core structure of large and extra-large colonies. Processed in Slicer 3D. Time between repetitions 350 ms and echo-time 4.66 ms. Time of acquisition 4 d 12 h 18 m, 29 averages. Resolution $23 \times 23 \times 23 \mu\text{m}^3$ captured with a matrix of $256 \times 196 \times 196$. Video length 10 s, 360 frames.

3 MR MICROSCOPY USING MICROCOILS AT 22 T: COIL CALIBRATION AND USAGE

Demonstrated on *Medicago truncatula* roots.

3.1 ABSTRACT

This chapter describes a method that was developed to calibrate microcoils designed for ultra-high field (22.3T), high-resolution magnetic resonance imaging (MRI). Microcoils increase sensitivity by matching the size of the RF resonator to the size of the sample of interest, thereby allowing higher image resolutions. Due to the relatively simple design, solenoidal microcoils can be custom made and adapted to the sample requirements. Systematically, I explain how to calibrate new or home-built microcoils, using a reference solution. The calibration steps include 1. pulse power determination using a nutation curve 2. Estimation of RF-field homogeneity and 3. calculating a volume normalised Signal-to-Noise Ratio (SNR) using standard pulse sequences. Important steps in sample preparation for small biological samples are discussed, as well as and possible mitigating factors such as magnetic susceptibility differences. The applications of an optimised solenoid coil are demonstrated by high-resolution ($13 \times 13 \times 13 \mu\text{m}^3$, 2.2 fL) 3D imaging of a *Medicago truncatula* root sample.

This chapter will be published as a video protocol:

van Schadewijk, R., Krug, J.R., Webb, A., van As, H., Velders, A.H., de Groot, H.J.M., Alia, A. (2019). Ultra-High Field Magnetic Resonance Microscopy using Microcoils: coil performance calibration and usage demonstrated on *Medicago truncatula* roots. (Manuscript submitted)

3.2 INTRODUCTION

Magnetic Resonance Imaging is a versatile tool to non-invasively image a wide variety of biological specimens, ranging from humans to single cells (Aguayo *et al.*, 1986; Ciobanu and Pennington, 2004; Radecki *et al.*, 2014). While MRI-scanners for medical imaging applications typically use magnets with a field strength of 1.5 T to 3 T, single-cell applications are imaged at much higher field strengths (Ciobanu and Pennington, 2004; Radecki *et al.*, 2014; Lee *et al.*, 2016). The study of specimens at resolutions below a hundred micrometres is referred to as Magnetic Resonance Microscopy (MRM) (Callaghan, 1993). However, MRM suffers from a low Signal-to-Noise Ratio (SNR) compared to other available microscopy or imaging techniques (*e.g.*, optical microscopy or CT). To optimise the SNR, several approaches can be pursued (Glover and Mansfield, 2002). One approach is to use a higher magnetic field strength, while a complementary approach is to optimise the signal detector for individual samples. For the latter, the dimensions of the detector should be adjusted to match the dimensions of the sample of interest. For small samples (≈ 0.5 -2 mm in diameter), *e.g.*, root tissues, microcoils are useful as the SNR is inversely proportional to the coil diameter (T L Peck, Magin and Lauterbur, 1995; Glover and Mansfield, 2002). Resolutions as high as $7.8 \times 7.8 \times 15 \mu\text{m}^3$ have been attained on animal cells using dedicated microcoils (Lee *et al.*, 2015). A variety of microcoil types exist, with planar and solenoid coils most commonly used depending on application and tissue geometry (Fratila and Velders, 2011). For example, a method designed specifically for imaging perfused tissue has been described for planar microcoils (Flint *et al.*, 2017). In this chapter, the characteristics of the solenoid coil are described as well as a protocol to prepare samples for microcoil MRI and the required steps to calibrate solenoid microcoils (Fig. 3.1A).

The solenoid coil consists of a conducting wire looped, like a corkscrew, around a capillary holding the sample. Used in combination with the specific MR system (Materials and Methods), microcoils can be constructed using only enamelled copper wire, an assortment of capacitors, and a suitable base for soldering the components (Fig 3.1B). The major advantages are the simplicity and low cost, combined with good performance characteristics in terms of SNR per unit volume and B_1 field homogeneity. The ease of construction enables fast iteration of coil designs and geometries. The specific requirements of solenoid microcoil design and probehead characterisation, *i.e.* the theory of electronics, workbench measurements and spectrometer measurements for a variety of coil geometries, have been described extensively elsewhere (T L Peck, Magin and Lauterbur, 1995; Haase *et al.*, 2000; Minard and Wind, 2001a, 2001b; Vegh *et al.*, 2012).

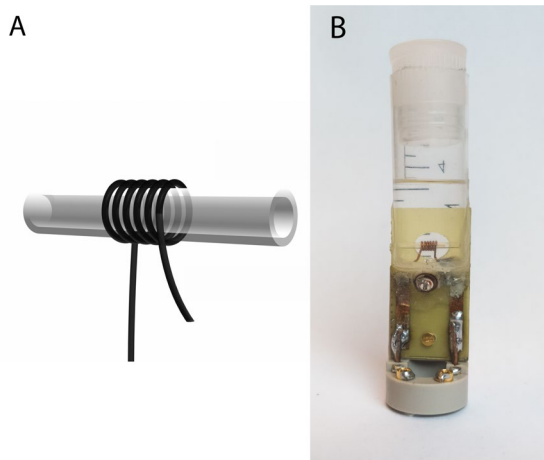


Fig. 3.1 A solenoid microcoil. (A) The solenoid coil design consists of wire looped helically, typically wrapped around a capillary. The geometry of the wire, such as its thickness, diameter, number of windings and wire spacing influence the coil characteristics. (B) A home-built solenoid microcoil with a reservoir for susceptibility matching fluid (Fomblin). It consists of a 0.4 mm thick coated copper wire wound six times around capillary with an outer diameter of 1500 μm . The coil is submerged in a reservoir which is made from a syringe. Sample capillaries up to an outer diameter of 1000 μm can be inserted. Two capacitors are used, a 1.5 pF capacitor in series with the inductor and a second variable 1.5-6 pF capacitor is placed in parallel to the inductor. All components are soldered to a fibreglass board (yellow). It is mounted on a commercial holder (grey polymer) that is modified to support the reservoir.

Basic experimental MR parameters are highly dependent on the hardware of the system used, including gradient system, field strength, and console. Several parameters can be used to describe the system performance, of which 90° pulse power, B_1 -homogeneity and SNR per unit volume (SNR/mm^3), are the most practically relevant. To this end, a standardised spin echo measurement using a phantom has been adapted to microcoils (Oerther, 2012). SNR/mm^3 is useful to compare the performance of different coils on the same system. While hardware differences across systems may exist, the uniform application of a benchmarking protocol also facilitates the comparison of system performance.

This protocol focuses on calibration and sample preparation. The stepwise characterisation of the performance of solenoid microcoils is shown: 1. calibrating the 90° pulse length and power 2. Assessing the RF-field homogeneity and 3. calculating SNR per unit volume (SNR/mm^3). A standardised spin echo measurement using a phantom is described to facilitate comparison of coil designs, which allows for optimisation of distinct applications. Phantom and biological specimen sample preparation, specific for microcoils, are described. The protocol may be implemented on any suitable narrow-bore (≤ 60 mm) vertical spectrometer equipped with a microimaging system. For other systems, it can serve as a guideline and can be used with some adjustments.

Biological specimen preparation for MRI measurements is usually not very extensive since the specimen is imaged as intact as possible. However, air spaces in biological tissue can cause image artefacts due to differences in magnetic susceptibility (Callaghan, 2007). The effect increases with increasing magnetic field strength (Donker *et al.*, 1997). Thus, air spaces should be avoided at high-field strengths, and this might require the immersion of the sample in a fluid to avoid air around the tissue and the removal of air spaces within the tissue structures. Specifically, when microcoils are employed, excision of the desired sample tissue might be required, followed by submerging it in a suitable fluid. This is followed by insertion of the sample into a pre-cut capillary, and finally sealing the capillary with capillary wax. Using wax as a sealant instead of glue, flame-sealing or alternatives, means that the sample may be easily extracted. This procedure is demonstrated on the root of *Medicago truncatula* a small leguminous plant. The advantage of co-registration of MRI data with optical microscopy is highlighted since the sample is not destroyed during the MRI measurement.

The presented protocol is suitable for high spatial resolution *in situ* measurements, and more elaborate designs could allow for imaging *in vivo* samples, where challenges related to life support systems would need to be addressed.

3.3 PROTOCOL

This protocol describes the necessary procedures to evaluate coil characteristics of a 1.5 mm inner diameter (ID) solenoid coil (Fig. 3.1B). The coil used to demonstrate the protocol is housed in a susceptibility-matched reservoir, but the protocol is equally applicable to unmatched coils. The protocol may be adapted to other sizes and different spectrometer setups.

A solenoid coil can be built by keeping in mind design rules for the desired dimensions according to the guidelines described elsewhere (Timothy L. Peck, Magin and Lauterbur, 1995; Webb, 2013). In this specific case, a coil was used with an inner diameter of 1.5 mm, made from 0.4 mm enamelled copper wire looped around a capillary of 1.5 mm outer diameter. This solenoid is held on a base plate on which a circuit is made, a tuning capacitor (2.5 pF), a matching capacitor (1.5-6 pF) as well as with copper connecting wires. The exact values of the capacitors are determined by the desired resonance frequency.

The susceptibility-matched design of the coil includes a reservoir with perfluorinated liquid to reduce susceptibility mismatches, arising from the copper coil being in close proximity to the sample (Olson *et al.*, 1995). A reservoir was made out of a plastic syringe to enclose the coil and filled with fomblin. As the perfluorinated liquid needs to enclose the coil, the available diameter for a sample is reduced to an outer diameter of 1 mm. For ease of sample changing, the sample was prepared in a capillary with an outer diameter of 1 mm and an inner diameter of 700 μm . The necessary tools for sample preparation are shown in Fig 3.2A.

The coil can be mounted on a holder which fits the Bruker Micro5 probe. In this case, a modified support insert was used, equipped with the necessary connections to connect to the ^1H channel of the Micro5 probe.

3.3.1. REFERENCE SAMPLE PREPARATION

3.3.1.1. To prepare 100 ml of the sensitivity reference solution, dissolve 156.4 mg of $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ into 80 mL of D_2O contained in a 100 mL GL45 flask. Manually stir until solids are completely dissolved.

3.3.1.2. Adjust volume to 100 ml using de-ionised water for a final concentration of $1 \text{ g L}^{-1} \text{ CuSO}_4$ (anhydrous).

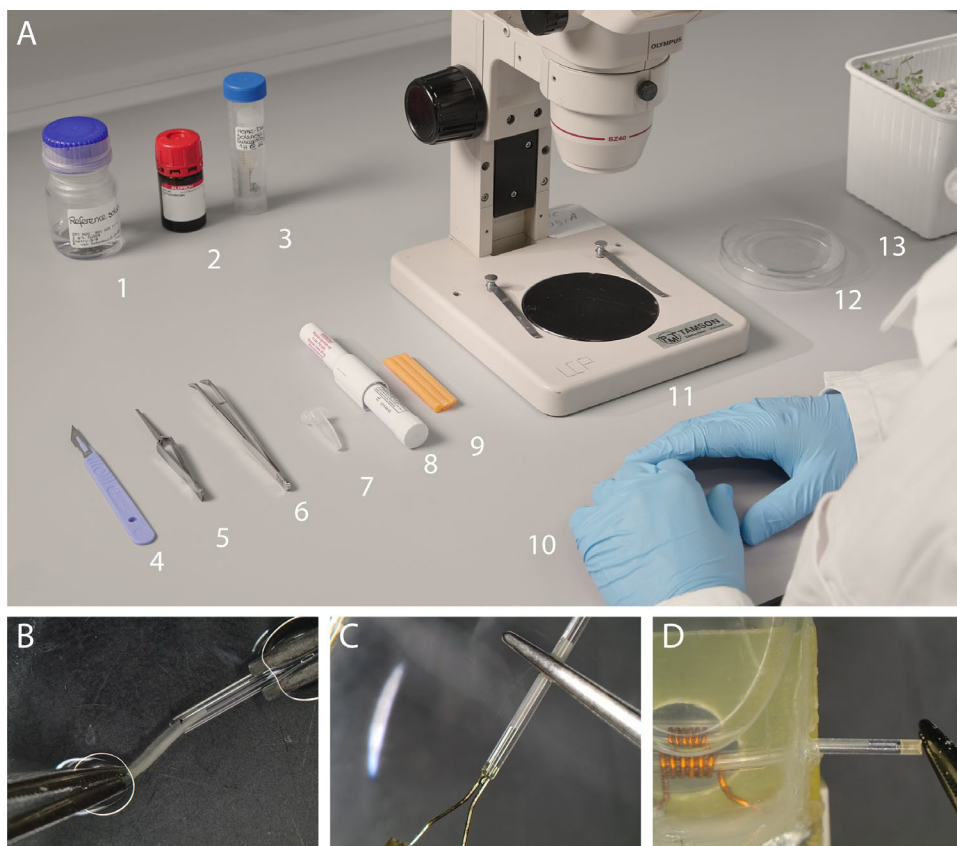


Fig. 3.2 Sample preparation under a stereomicroscope. (A) Items needed for the preparation of microcoils. From left to right: 1. CuSO_4 reference solution, 2. perfluorodecalin, 3. microcoil, 4. scalpel, 5. positive tension tweezers, 6. tweezers, 7. capillaries outer diameter = $1000 \mu\text{m}$, 8. wax pen, 9. capillary wax, 10. nitrile gloves, 11. stereomicroscope, 12. watch glass with petri dish cover, 13. plant material in growth substrate. Not shown: 2 mL syringe with $\varnothing 0.8 \times 40 \text{ mm}$ needle and fine tissue paper. (B) Close up of sample insertion into a capillary using tweezers, while both are kept submerged. (C) Sealing of the capillary using molten wax. (D) Insertion of the prepared capillary into the microcoil.

NOTE: The reference sample should be sealed to prevent changing the ratio of H₂O: D₂O.

3.3.2. SAMPLE PREPARATION

- 3.3.2.1. If preparing a reference sample, add 1 ml CuSO₄ solution to a watch dish under a stereomicroscope.
- 3.3.2.2. If preparing a biological sample, place 1 ml of perfluorodecalin (PFD) inside a watch glass under a stereomicroscope. PFD will infiltrate a biological specimen without entering the intact cells and thereby reduce the number of air cavities. Immediately cover the watch glass with a petri dish lid to prevent evaporative loss, until the PFD is needed. NOTE: PFD is highly volatile and a potent long-term greenhouse gas (Tsai, 2011). When its oxygen-dissolving properties and its low viscosity are not required, it may be substituted with Fomblin, a perfluoroether which also gives no observable ¹H signal, but which does not evaporate as quickly (Olson *et al.*, 1995).
- 3.3.2.3. Capillaries of suitable outer diameter need to be cut to size, to fit inside the diameter of the microcoil holder (18 mm) and allow for repositioning (Fig. 3.1B). Use a ceramic cutter to make an incision every 10-12 mm and break carefully on the incision point.
- 3.3.2.4. If preparing a reference sample, use tweezers and the stereo microscope, to bring a pre-cut capillary in contact with the surface of the CuSO₄ solution inside the watch glass, allowing capillary action to fill the capillary.
- 3.3.2.5. If preparing a biological sample, use tweezers and a stereo microscope, to bring a pre-cut capillary in contact with the surface of the PFD inside the watch glass, allowing capillary action to fill the capillary fully. Release the capillary into the watch glass so that it becomes fully submerged.
 - 3.3.2.5.1. Next, carefully extract a whole root system from its soil or soil replacement. Clean the root sample meticulously. Photograph if needed for future reference. Select and excise a root section using a scalpel.

To reduce the presence of air pockets within biological samples:

- 3.3.2.6. Place the sample into a 1.5ml Eppendorf tube containing a suitable buffer solution. Leave the tube cap off, then apply parafilm to seal the opening of the tube. Then, punch holes with a sharp tool to allow for ventilation of the tube.
- 3.3.2.7. Place the sample tube in a vacuum chamber, seal the chamber, connect a vacuum pump to the chamber and apply vacuum for 30 minutes. Air bubbles may be seen escaping the sample.
- 3.3.2.8. Using tweezers and stereomicroscope, submerge the sample in the infiltration medium prepared previously. Wash the sample of potential debris.
- 3.3.2.9. Next, insert the sample into the capillary using tweezers, while both capillary

and sample are fully submerged in order to avoid inclusion of air bubbles. A smaller capillary or syringe may serve as a pushing rod (Fig. 3.2B).

- 3.3.2.10. Take the sample capillary from the watch glass, using tweezers. In the case of PFD, cover the petri-dish lid.
- 3.3.2.11. Remove circa 1 mm of liquid from both ends of the capillary using fine tissue paper.
- 3.3.2.12. Melt a small volume of capillary wax using a wax pen. Apply wax on either side while taking care to eliminate any possible air pockets with the capillary (Fig. 3.2C). When the wax has hardened, it will become opaque.

NOTE: Avoid overheating wax or capillary as this may cause explosive boil off as well as cavitation pockets when the finished sample cools.

- 3.3.2.13. Remove excess wax from the exterior of the capillary with a scalpel and wipe clean with fine tissue paper.

3.3.3. MOUNTING SAMPLE

- 3.3.3.1. Place a microcoil underneath the stereo microscope and insert the sample using tweezers while immobilising the microcoil (Fig. 3.2D).
- 3.3.3.2. Position the sample in the centre of the coil by sliding the capillary inside the solenoid coil.
- 3.3.3.3. Optionally, apply scotch tape to fix the position of the capillary.
- 3.3.3.4. Inspect the capillary to ensure no air bubbles are visible inside the solenoid coil, to avoid MR signal destruction caused by susceptibility differences.
- 3.3.3.5. Place the microcoil on the socket of the probe base (Fig. 3.3A & B).
- 3.3.3.6. Carefully slide the gradient coils over the microcoil while matching the water-cooling connectors of the gradient to that of the probe base (Fig. 3.3C). (Note: This step applies for a Micro5 probe only. In the case of a Micro2.5 or a Biospect system, the gradients are on a separate socket than the coil)

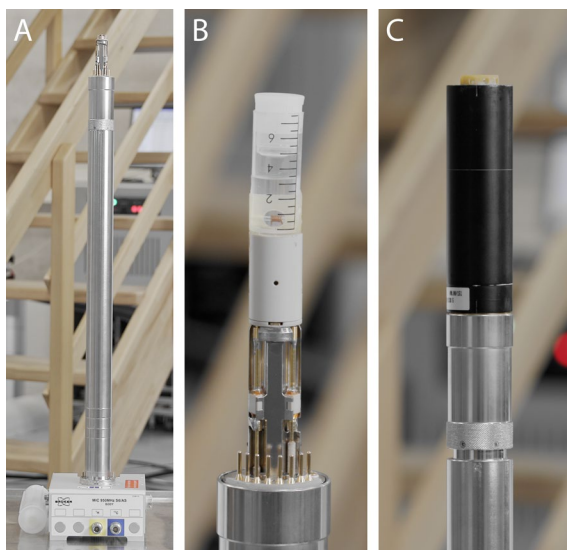


Fig. 3.3 The components of a micro-imaging probe. (A) Micro5 probe base, containing all necessary connections for water cooling, heating, temperature sensors, gradient power, RF (co-axial connector visible) and optionally probe identification (PICS). Underneath the probe base are knobs that allow for adjusting the variable tuning and matching capacitors, as well as retaining screws to hold the probe in place inside the spectrometer. (B) The home-built microcoil mounting atop the probe-base. Note the variable capacitors (white ceramic) mounted on the probe-base that allow for tuning and matching. (C) Integrated 3-axial gradient mounted on the probe base with water-cooling receptacles and gold-plated contacts for grounding the gradient.

3.3.4. DETERMINING COIL CHARACTERISTICS

If the coil is tested for the first time, it is recommended to use the reference sample solution to create a homogeneous sample, which is useful for power calibration and B_1 homogeneity tests. Also, potential susceptibility problems due to the coil wires may be tested easily with this reference sample.

- 3.3.4.1. To test if the coil resonates at the desired resonance frequency, the probe base can be connected to a network analyser. An S_{11} test can be performed to test the frequency range achieved by tuning and for Q-factor measurements as described in Haase *et al.* (Haase *et al.*, 2000).
- 3.3.4.2. Insert the probe into the magnet and connect all cables.
- 3.3.4.3. Set desired water-cooling temperature (recommended 298 K) for the water cooling unit (BCU20).
- 3.3.4.4. Set the target temperature (298 K) and the target gas flow (300 L h^{-1}). The gas flow might be different for a different coil design or sample volume. This applies only to systems with a temperature control system.

NOTE: The next steps are only necessary when test novel (home-built) coils.

- 3.3.4.5. Connect the probe using a 50 Ω co-axial cable to a network analyser with a suitably wide sweep width (400 MHz), centred on the intended resonance frequency.
- 3.3.4.6. Observe the resonant modes by adjusting the matching and tuning capacitors of the probe base.
- 3.3.4.7. Tune and match the resonant mode to the desired frequency.
- 3.3.4.8. Optionally, determine the coil quality factor (Q-factor) on a network analyser. One method to obtain the quality factor is to use a coupling network and dividing the centre frequency by the width of the resonance peak at -3 dB (*i.e.*, $Q = 2 * f_c / (f_1 - f_2)$) (Haase *et al.*, 2000). Some network analysers have Q-factor determination built-in.
- 3.3.4.9. Initiate a wobble curve and adjust the tuning and matching as necessary. It is recommended to set any tuning and matching capacitors to the midpoint of their range for new coils. Therefore, using a high spectral sweep width is recommended. In some cases, it might be more convenient to tune and match the coil outside the magnet on a network analyser.
- 3.3.4.10. Select a shim file for the largest volume coil of the imaging probe if it is available. If starting from a coil which has been used previously, use an available shim file. If both options are not available, start with all shim values set to 0.
- 3.3.4.11. Select correct coil configuration if it is available in the imaging software (*i.e.*, ParaVision). Else, create a new coil configuration matching the specifications of the coil, *e.g.*, single tuned or double tuned, according to the manual of your system. Estimations for the safe limits for this solenoid microcoil used in this research with 1.5 mm inner diameter in size is 1 ms at 1W peak power and 1 mW continuous power.

CAUTION: The small capacitors (typically 1 mm in size) needed for microcoils are highly sensitive and easily damaged by high voltages. Automated pulse power determination might not function with non-standard coils, and too high powers could cause damage to the coil or other parts of the spectrometer. Therefore, manual adjustments are recommended.

- 3.3.4.12. A nutation curve should be recorded for a new coil to obtain an indication of the correct rf-power for the coil (Fig. 3.4). Using an FID-experiment in the absence of gradient encoding, the RF-pulse length is varied systematically while the pulse power is kept constant. The ideal pulse length is the pulse length where the signal intensity reaches the maximum. If testing a new coil, use a 10 μ s pulse with a very low power first and start increasing the pulse power gradually. In case the power is much higher than expected for the

combination of coil characteristics and spectrometer, this is already an indication that the wrong resonant mode has been selected. For a coil with a homogeneous B_1 -field, like a solenoid coil, the 180° pulse can also be determined where the signal intensity decreases to zero (Keifer, 1999).

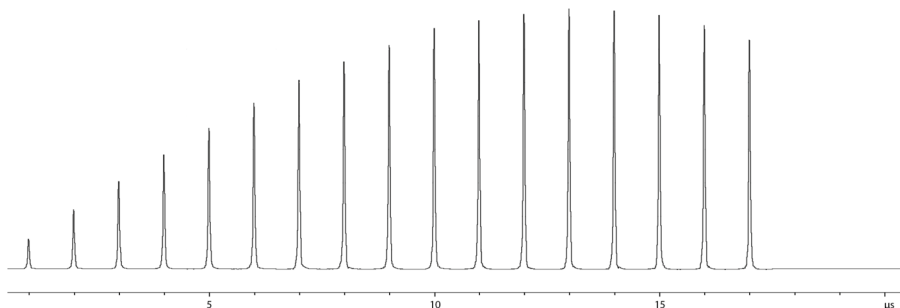


Fig. 3.4 Nutation curve. A nutation curve is acquired to determine the reference pulse power. The reference pulse power (90° pulse) is defined as the combination of power and pulse length needed to generate a B_1 field that flips all available magnetisation in the z-direction to the transverse plane. A series of a pulse is recorded in the absence of gradient encoding. With each pulse, either pulse length or pulse power is incremented. Here the pulse power is set to 0.6 W, while the pulse length is incremented by 1 μ s each time. The maximum signal intensity indicates the 90° pulse, around 12-13 μ s. The 180° pulse may also be determined in this way using the minimum intensity.

- 3.3.4.13. Set the determined 90° pulse power into the adjustment card of the created study (In ParaVision, the reference power adjustment card may be used to enter the hard pulse power).
- 3.3.4.14. Use a Localiser scan with 3 slices, one slice in each of the three primary axes, to locate the position of the coil within the magnet. Starting with a large field-of-view is recommended. If the sample is exactly in the centre of the gradient system, the Localizer scan will show the sample. If the coil or sample is not centred in the image slices or missing, the localiser scan might need to be adjusted.
- 3.3.4.15. A complementary way to find the correct 90° pulse is based on image evaluation: Once an approximate pulse power is found, one could adjust the pulse powers gradually to check the image for B_1 -field homogeneity. For some coils, the 90° pulse power determined using the nutation curve could be overestimated, which leads to over-tipping in the homogeneous region of the B_1 field. The pulse reference pulse may then be reduced, and the new images can be checked against the previous images (Fig. 3.5).
- 3.3.4.16. Manually shim the magnetic field based on the FID signal. A recommended order for initial shimming is Z-Z²-Z-X-Y-Z-Z²-Z-XY-XZ-YZ-Z. In the case of a solenoid, the main symmetry-axis is in the XY-plane. Therefore, shims in different directions might be more sensitive for this coil configuration. Higher

order shims have little effect and may be ignored.

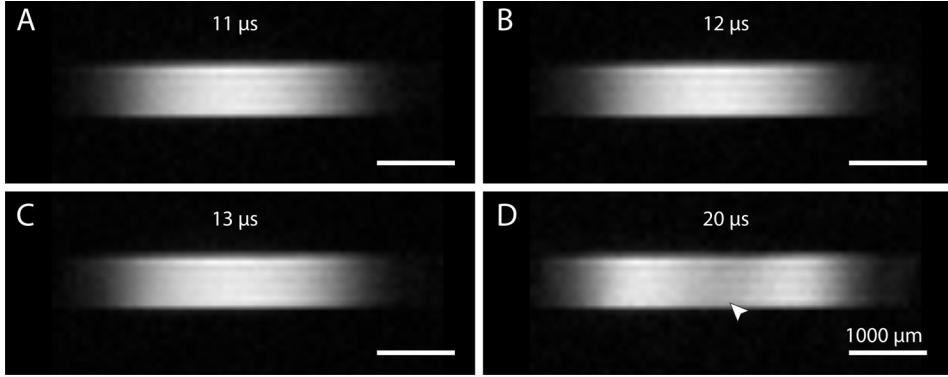


Fig. 3.5 Visual determination of 90° pulse power Once an approximate reference pulse power has been found using a nutation curve, it may be checked visually by varying the pulse length. Depending on the coil, the B_1 field may be more or less sensitive to changes. (A) 11 μ s pulse length. (B) 12 μ s pulse length, optimal for this coil. (C) 13 μ s pulse length. (D) 20 μ s pulse length. If the pulse power is set too high, over-tipping may occur, thereby reducing image intensity in the centre of the coil (arrowhead). The increased B_1 field also increases the range of the coil, as can be observed in the width of the image.

3.3.4.17. Next, a volume-normalised SNR must be calculated to allow for comparison of coil characteristics across different systems, adapted from the manufacturer's protocol (Oerther, 2012). A spin echo sequence is used with the following parameters: FOV 6 mm x 6mm TR 1000 ms, TE 7 ms, Matrix 256 x 256 and Slice thickness = 0.5 mm. Adjust the slice thickness until the receiver gain is unitary. Next, adjust the number of slices so that slices extend beyond the region of B_1 -field homogeneity. Record the images without signal averaging if possible.

3.3.4.18. The volume normalised SNR (SNR/mm^3) can be determined in two steps. First, the Voxel volume (V_{voxel}) is calculated (Eq. 3.1):

$$V_{\text{voxel}} = D_x \times D_y \times D_{\text{slice}} \quad (3.1)$$

Then, by selecting regions of interest, determine the signal intensity (μ) and standard deviation (σ) of the sample (*i.e.*, the signal) and a region outside the sample (*i.e.*, the noise). Either the spectrometer control software or general-purpose image processing software may be used for these calculations. The values may then be used to calculate a volume normalised SNR (Eq. 3.2):

$$\text{SNR}_v = \frac{\mu_{\text{ROI}} - \mu_{\text{noise}}}{\sigma_{\text{noise}} \times V_{\text{voxel}}} \quad (3.2)$$

When comparing the SNR of coils at different magnetic field strengths, the relaxation properties would need to be taken into account (Vlaardingerbroek and Boer, 2013).

NOTE: The units for D_x , D_y and D_{slice} are in mm. This calculation can likewise be performed for a series of slices.

3.3.4.19. Check for susceptibility problems due to magnetic field inhomogeneities: load and run a multiple gradient-echo (MGE) sequence (Fig. 3.6). Magnetic field inhomogeneities due to susceptibility differences are visible in the images with higher echo times as the gradient echo does not refocus spins which dephase due to static field inhomogeneities. This way inhomogeneities in the sample may be visualised (due to, *e.g.*, air spaces in the sample), as well as B_0 field inhomogeneities introduced by the coil material. The following parameters are suggested, to be adjusted depending on the specifications of the spectrometer and coil used: repetition time 200 ms, echo time 3.5 ms with 48 echoes spaced 3.5 ms apart, flip angle 30 degrees. Matrix size 128×128 .

NOTE: If multiple resonant modes were observed in the resonance (wobble) curve, repeat the above steps for each resonant mode to determine the most sensitive one.

3.3.5. HIGH-RESOLUTION IMAGING

3.3.5.1. Run a 3D-FLASH experiment with the following suggested parameters: Repetition time 70 ms, echo time 2.5 ms, matrix size of $128 \times 64 \times 64$, field of view $1.6 \times 0.8 \times 0.8$ mm, and receiver bandwidth 50 kHz. Adjust the FOV, if necessary, to cover the whole object in both phase encoding directions, thereby avoiding aliasing.

3.3.6. RECOVERING SAMPLES FOR FURTHER STUDY OR STORAGE

3.3.6.1. Remove the sample capillary from the microcoil.

3.3.6.2. Using tweezers, remove the wax plugs under a stereomicroscope.

3.3.6.3. A syringe may be used to wash the sample out of the capillary with a solution of choice. Alternatively, a glass pusher rod may be used to eject the sample.

3.3.6.4. To prevent dehydration of the sample, store in a suitable medium for storage.

3.4 REPRESENTATIVE RESULTS

3.4.1 COIL CHARACTERISATION

Upon successful tuning and matching of a coil, its performance may be characterised by the coil Q-factor, 90° reference pulse, and SNR/mm³. For the 1.5 mm ID susceptibility-matched solenoid coil demonstrated here, the Q-factor(unloaded) was 244, compared to 561 for a 5 mm birdcage coil.

The reference 90° pulse was 10-12 μs at a power level of 0.6W; *cf.* a 5mm birdcage coil used in the same spectrometer was 5 μs at 45 W. See also Fig. 3.5. The most relevant to image quality, however, is the SNR_v for the microcoil. For the coil used here, filling in Eq. 3.2 results in the following:

$$\text{SNR}_v = \frac{2.3 \cdot 10^1 - 3.4 \cdot 10^{-1}}{1.8 \cdot 10^{-1} \times 2.75 \cdot 10^{-4} \text{ mm}^3} = 4.6 \cdot 10^5 \text{ mm}^{-3} \quad (3.3)$$

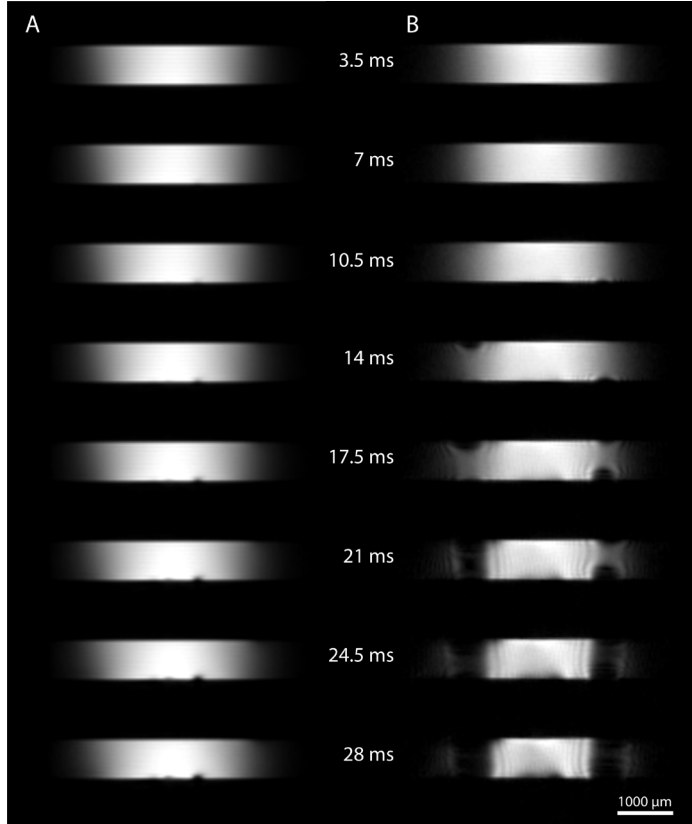


Fig. 3.6 RF homogeneity evaluated by gradient echo imaging. A Multiple Gradient Echo (MGE) sequence is used to evaluate RF (B_1 -Field) homogeneity using a series of gradient echoes. Basic parameters were: repetition time 200 ms, echo time 3.5 ms with number of echoes 48, echo spacing 3.5 ms, 64 averages, acquisition time 27 m 18 s, flip angle 30 degrees. Field of view was 5×5 mm, matrix 128×128 , resolution $39 \times 39 \times 200 \mu\text{m}^3$. **(A)** Susceptibility-matched coil. The susceptibility matching fluid (Fomblin) surrounding the RF coil reduces susceptibility effects due to the coil wire. Small air bubbles cause loss of signal as the echo time increases. **(B)** A coil (not susceptibility matched) with equal coil diameter. At longer echo times increasing artefacts caused by B_0 field inhomogeneity are observed.

3.4.2 EFFECT OF SUSCEPTIBILITY MATCHING

At ultra-high field strengths, sample and coil susceptibility become a dominant factor for image quality as seen in Fig. 3.6A and B. Compared to a coil lacking a susceptibility matching fluid reservoir, the signal is retained longer and more homogeneously in a reference sample. However, due to the susceptibility reservoir, the maximum sample dimensions decrease with respect to the coil without the reservoir.

3.4.3 HIGH-RESOLUTION IMAGING

A high resolution of $13 \times 13 \times 13 \mu\text{m}^3$ of a *Medicago truncatula* root specimen was attained in 20 hours and 23 minutes (Fig. 3.7). Starting from the surface of the root, the root cortex is seen, along with some residual water on the outside of the root. Furthermore, the xylem is observed as a dark band enclosing the phloem. Some air pockets are observed as dark spots with near-complete signal loss.

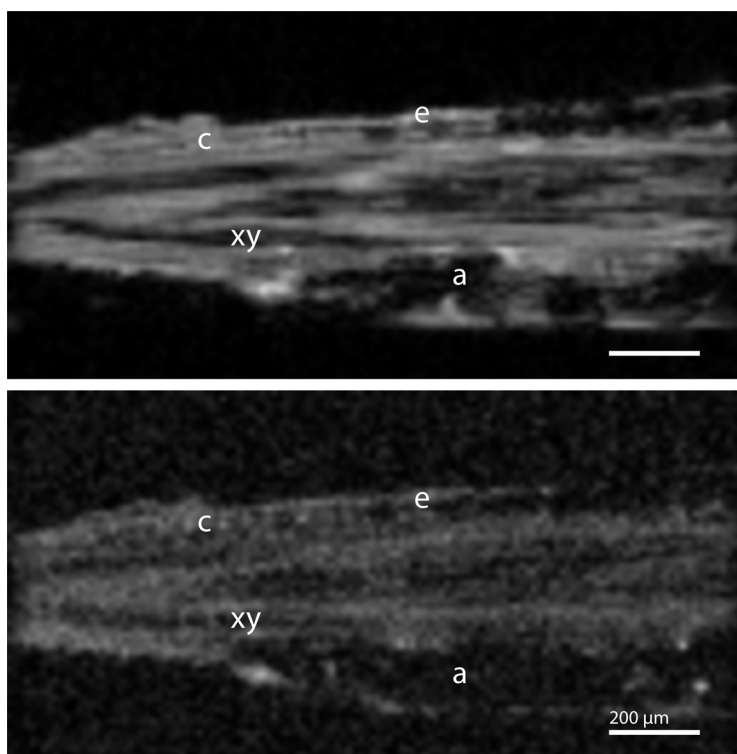


Fig. 3.7 3D imaging of a *Medicago truncatula* root section. **(A)** FLASH image. Several features of the root section can be distinguished, including the epidermis (e), cortex (c) and xylem (xy). Air pockets (a) in the root cause complete signal loss. Basic parameters were as follows: Repetition time 70 ms, echo time 2.5 ms, 256 averages, acquisition time 20 h 23 m. Resolution $13 \times 13 \times 13 \mu\text{m}^3$. Matrix size was $128 \times 64 \times 64$ and field of view $1.6 \times 0.8 \times 0.8 \text{ mm}$. Receiver bandwidth 50 kHz. **(B)** MSME image. Basic parameters were as follows: Repetition time 500 ms, echo time 5.2 ms, 28 averages, acquisition time 15 h 55 m. Resolution $13 \times 13 \times 13 \mu\text{m}^3$. Matrix size was $128 \times 64 \times 64$ and field of view $1.6 \times 0.8 \times 0.8 \text{ mm}$. Receiver bandwidth 70 kHz.

3.5 DISCUSSION

Even for simple circuit designs, multiple resonant modes can be present, especially near capacitors close to the solenoid coil. These alternate modes represent a pathway for RF transmission loss and reduce the overall sensitivity of the coil. Identifying the primary resonant mode is therefore important but can be affected strongly by the properties of the probe to which it is mated. Hence, coils have to be developed in conjunction with the probe base which contains adjustable tuning and matching capacitors.

As microcoils are ideally very close to the sample, the magnetic susceptibility differences between the medium and the wire can cause additional signal loss. For this reason, we presented a susceptibility-matched coil, by submerging the wire in fluorinert liquid (Fomblin or FC-43). An alternative approach for constructing a susceptibility matched coil is to use susceptibility- matched wire (Kc *et al.*, 2010). Furthermore, only susceptibility issues due to the coil are addressed with this approach. Susceptibility mismatches inside the sample (*e.g.*, due to air spaces) remain challenging.

Air pockets or bubbles pose an experimental challenge that causes extensive signal loss, caused by susceptibility differences at the interface of the air and the fluid or specimen (Callaghan, 2007) (Fig. 3.5A). An important aspect for successful sample preparation is the submersion of both sample and capillary. However, even small bubbles can cause signal losses, especially for gradient echo type sequences. Mobile air bubbles can migrate through the capillary until they are in contact with the sample. Some of these effects can be alleviated by slightly tilting the capillary so that one end is higher than the other. Tilting ensures potential air bubbles are held in place at the higher end, without disturbing the sample.

For the air spaces inside the sample, PFD was used to fill up the intercellular air spaces (Littlejohn *et al.*, 2010) while not penetrating the cell membranes. However, even with this approach, we were not able to remove all air spaces. Additionally, this approach means that we need an additional agent, which is usually not preferred due to the desire to study a system as non- invasive as possible.

The cylindrical shape of capillaries means that perfusion setups should be viable, especially for tissues vulnerable to decay such as biopsies or studying processes in living root material. Two steps could realise a perfusion setup. First, by connecting a medium feed tube and drain tube at either side of the capillary would be sufficient to create a chemostat. Second, the addition of an indentation in the sample capillary could hold the sample in place against the direction of flow. This is analogous to a protocol published for planar microcoils (Flint *et al.*, 2017).

The non-invasive nature of MR imaging, combined with the inert liquid used in this protocol (PFD or Fomblin), means that after completion of experiments samples may be removed from their capillaries for further study. Combinations include optical or electron microscopy and other destructive imaging techniques.

We have demonstrated a method for imaging plant material using dedicated microcoils on an ultra-high field NMR spectrometer. Relatively large sample volumes can be studied at high resolution with RF homogeneity. Adapting microcoil design to samples is facilitated by an efficient method to determine coil performance characteristics. The solenoid coil approach may also be readily applied to other samples than plants, including animal tissue.

3.6 ACKNOWLEDGEMENTS

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4 MR MICROSCOPY OF *MEDICAGO TRUNCATULA* AT 22.3 TESLA

Cellular Resolution and Localised Spectroscopy

4.1 ABSTRACT

Interactions between plants and the soil's microbial & fungal flora are crucial for the health of soil ecosystems and food production. Microbe-plant interactions are difficult to investigate *in situ* due to their intertwined relationship involving morphology and metabolism. Here, an approach is described to overcome this challenge by elucidating morphology and the metabolic profile of *Medicago truncatula* root nodules using Magnetic Resonance (MR) Microscopy, at the highest magnetic field strength (22.3 T) currently available for imaging. A home-built solenoid MR signal detector with an inner diameter of 1.5 mm was used to study individual root nodules. A 3D imaging sequence with an isotropic resolution of $(7\ \mu\text{m})^3$ was able to resolve individual cells, and distinguish between cells infected with rhizobia and uninfected cells. Furthermore, the metabolic profile of cells in different sections of the root nodule was studied using localised MR spectroscopy, showing that several metabolites, including betaine, asparagine/aspartate and choline, vary across nodule zones. The metabolite spatial distribution was visualised using chemical shift imaging. Finally, the technical challenges and outlook towards future *in vivo* MR microscopy of nodules and the plant root system are described.

This chapter is based on:

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4.2 INTRODUCTION

Interactions between plants and microbes are considered to be crucial for the health of the soil ecosystem as a whole (Doran and Zeiss, 2000). Understanding the metabolic interactions between plants and microbes, both commensal and parasitic, could help address many of the challenges we face today, related to agriculture and food security. One such interaction is the microbiome-mediated uptake of nitrogen by plants. Availability of biologically-active forms of nitrogen in the soil is one of the most crucial factors determining crop yield. Current agricultural practice, therefore, relies strongly on nitrogen fertiliser to supplement soil nitrogen to ensure high crop yield (Smil, 2001). The Haber-Bosch nitrogen fixation process, used to produce the ammonia needed for these fertilisers, currently consumes 1% of the world energy sources; making it the most energy consuming process in the chemical industry (Smith, 2002). In contrast, alternative processes (e.g. symbiotic nitrogen fixation, SNF) achieve the same result of fixing nitrogen without the need for high pressure and temperature required by the Haber-Bosch process.

More precisely, plants have solved the problem of biological nitrogen fixation through commensal processes, involving bacterial infection of plant roots (Suzaki, Yoro and Kawaguchi, 2015). Mutualistic infections are omnipresent in nature, with a wide range of nitrogen-fixing bacteria – such as those that are collectively named rhizobium – invading not just plants but also the phycosphere of green algae (Kim *et al.*, 2014). The mutualistic symbiosis involves almost all parts of the plant cell machinery, including plastids and mitochondria. Of particular interest are the interactions between rhizobial bacteria and leguminous plants, which form dedicated organs - root nodules - to accommodate the bacteria. *Medicago truncatula* infected with *Sinorhizobium meliloti* (*S. meliloti*) has long since been used as a model plant system to study plant-rhizobia interactions (Barker *et al.*, 1990). The root nodules facilitate intracellular hosting and nutrient exchange (Nap and Bisseling, 1990). The result is the efficient formation of fixed nitrogen (NH_4^+), which provides considerable growth advantages to leguminous plants (Udvardi and Day, 1997). Recently, a detailed fate map of root nodule formation at a genetic level has been developed (Xiao *et al.*, 2014). It has also been proposed that rhizobial metabolic activity may confer resistance to drought and salt stress (López, Tejera and Lluch, 2009; Kunert *et al.*, 2016; Staudinger *et al.*, 2016). More detailed understanding of key metabolites in plant-nodule metabolism and importantly, their localisation within root nodule tissues could, therefore, shed light on the mechanism by which Symbiotic Nitrogen Fixation (SNF) confers advantages to host plants.

Tracking metabolite exchange non-invasively within intact nodule systems is difficult with most imaging modalities, since they require a form of sample fixation, for example in the case of Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry (MALDI-MS) imaging (Ye *et al.*, 2013). The current knowledge of the metabolic profile in the root nodules is based on analysis with destructive extraction procedures. For example, Gas Chromatography and Liquid Chromatography in combination with Mass Spectrometry

(GC/LC-MS) has provided rich metabolic information but required destructive extraction procedures (Ogden *et al.*, 2017). These *in vitro* methods may not faithfully reflect the native structural and molecular information. Examining and mapping the levels of metabolites directly in the intact root nodule, non-invasively, would be important to understand the functional framework of metabolism in the native state. Magnetic resonance techniques may offer advantages in terms of spatially resolved spectroscopic information on a single nodule, which is difficult to access with alternative techniques. Magnetic resonance imaging (MRI) has been previously used to visualize belowground root structures (Metzner *et al.*, 2014; Schmittgen *et al.*, 2015; van Dusschoten *et al.*, 2016). In this chapter, state-of-the-art magnetic resonance microscopy (MRM) is applied in conjunction with localised spectroscopy at ultra-high magnetic field (22.3T), using a home-built solenoid RF coil, in order to image root nodules of *Medicago truncatula*. Not only cellular level structural information is obtained but also a mapping of metabolic information in localised zones, and how they vary across different tissues. Lastly, I describe the technical challenges and outlook towards future *in vivo* imaging of nodules and plant root systems.

4.3 RESULTS

4.3.1 ROOT NODULE MORPHOLOGY RESOLVED BY MR MICROSCOPY IN CELLULAR DETAIL

A representative *M. truncatula* specimen with a single root nodule shown in Fig. 4.1A, was imaged using a custom home-built 1.5 mm diameter solenoid coil as depicted in Fig. 4.1B.

Figure 4.1 Photograph of *M. truncatula* root system and custom-designed home-built microcoil. (A) Photograph of a representative *M. truncatula* plant (five weeks old, three weeks post inoculation). Typical root nodule indicated with a red square. Some white perlite beads are still visible along the root system. (B) Photograph of a home-built solenoid coil insert mounted on a Bruker insert holder. The solenoid inner diameter is 1.5 mm; further details are described in the materials and methods.



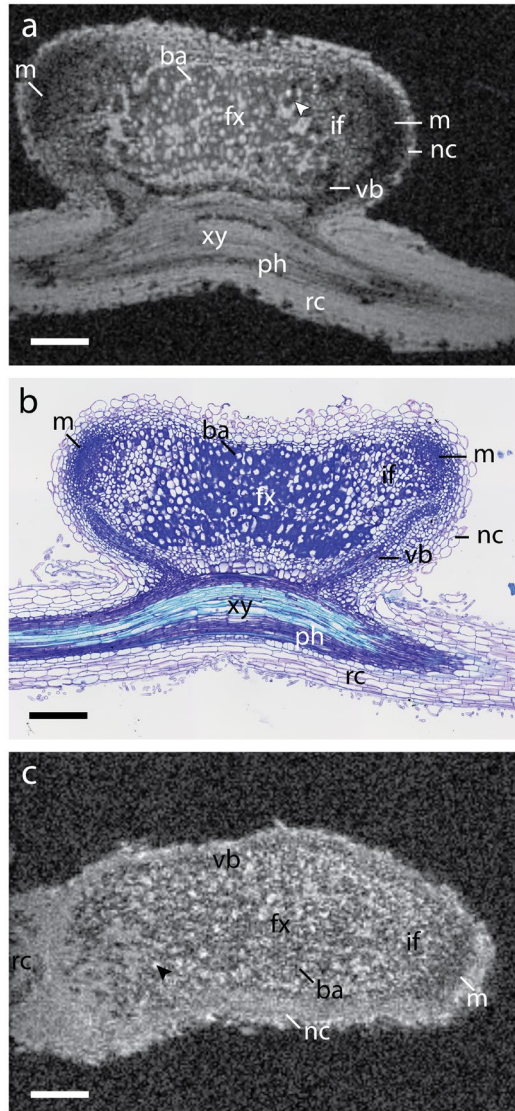


Figure 4.2 High-resolution MR imaging and optical microscopy of root nodules. **(A)** FLASH image of fixed root nodule at $7 \times 7 \times 7 \mu\text{m}^3$ resolution. Individual cells containing bacteroids (ba) can be discerned by the dark rings inside the cells, where *S. meliloti* cells accumulate. The nodule exhibits a double meristem (m) on opposed directions with cells becoming smaller towards the meristem until individual cells can no longer be resolved. Air pockets appear as hypo-intense regions marked with an arrowhead. **(B)** Optical transmission microscopy of nodule section (thickness $5 \mu\text{m}$) stained with Toluidine blue. **(C)** FLASH image of *in vivo* root nodule at $7 \times 7 \times 7 \mu\text{m}^3$ resolution. Though lower in signal-to-noise, both uninfected cells (black arrow) and infected cells (ba) can be discerned. Abbreviations: rc, root cortex; xy, xylem; ph, phloem; nc, nodule cortex; vb, vascular bundle; fx, fixation zone; if, infection zone; ba, bacteroid containing cells; m, meristem. Scale bars $200 \mu\text{m}$.

Individual cells of a root nodule with two lobes and meristems (m) could be distinguished on the MRI scans. A high-resolution 3D Fast Low Angle SHot (FLASH) image of the fixed root nodule is shown in Fig. 4.2A. All image slices through the entire root nodule are shown in the supplementary material (Supplementary Video 4.S1). Root nodule morphology in *M. truncatula* nodules are of the indeterminate type, *i.e.*, four distinct regions (zones) occur, from the apex of the root nodule to the root attachment point (Suzaki, Yoro and Kawaguchi, 2015). Nodule tissue - from outward to inward - starting with the nodule cortex (nc), delineated as a high-intensity ring on the surface of the nodule, contrasted with the darker region of the meristem (m). Cells in the meristem, responsible for the growth of the nodule, were significantly smaller than mature bacteroid containing cells. In the next region, the rhizobial infection zone (if), young cells infected by rhizobia bacteria could be seen as a region of alternating high- and low-intensity patches. Not all cells in the rhizobial infection zone were infected, which was apparent due to the presence of variation in image intensities, *i.e.*, non-infected plant cells had a higher image intensity as compared to cells infected with the bacterium. Lastly, in the active nitrogen fixation zone (fx), large cells (40 to 50 μm) could be distinguished by their grey rings, caused by the presence of bacteroids surrounding the vacuole (Gavrin *et al.*, 2014). The fixation zone is where rhizobia make nitrogenase, the enzyme required for nitrogen fixation. In the same area, uninfected interstitial cells were visible, which did not exhibit the rings. These interstitial cells perform an important role in the regulation of nitrogen fixation activity (Dakora and Atkins, 1991). Lastly, connective tissues (vascular bundles) could be seen extending from the root towards the periphery of the nodules, providing a route for nutrient exchange with the rest of the plant.

Fig. 4.2B shows a light microscopy image of a histological slice stained with Toluidine blue, from the same root nodule at approximately the same plane as the MR image (co-registration). Toluidine blue stained the nuclei and lignin of the cell wall (O'Brien, Feder and McCully, 1964). Common features in Fig. 4.2A and 4.2B are seen, including the bacteroid ring as observed on the MR image, occurring in the staining of cells in the nitrogen fixation zone. Especially visible were the vascular bundles extending from the central root, which due to their smaller cells appeared more densely stained in the optical microscopy image. In addition, air pockets could also be imaged as they appeared as hypo-intense regions in the FLASH image (Fig. 4.2A, arrowhead). Air pockets also introduced differences in magnetic susceptibility within the sample, which influenced image quality. Air pockets of different sizes could be seen within the fixation zone using multiple gradient echo (MGE) imaging with echoes acquired at increasing echo times (Supplementary Fig. 4S2). Because of the abundance of air pockets in the apical zone, the susceptibility-induced hypointense regions also increased in size in the apical direction.

To evaluate the possibilities of high-resolution MR imaging of root nodule without any fixation or treatment, measurements were performed on freshly excised nodules (Fig. 4.2C). While the image contrast and signal-to-noise ratio (SNR) was noticeably reduced,

individual cells in the nitrogen fixation zone could still be distinguished. The uninfected cells appeared bright while infected cells appeared dark in the active infection zone.

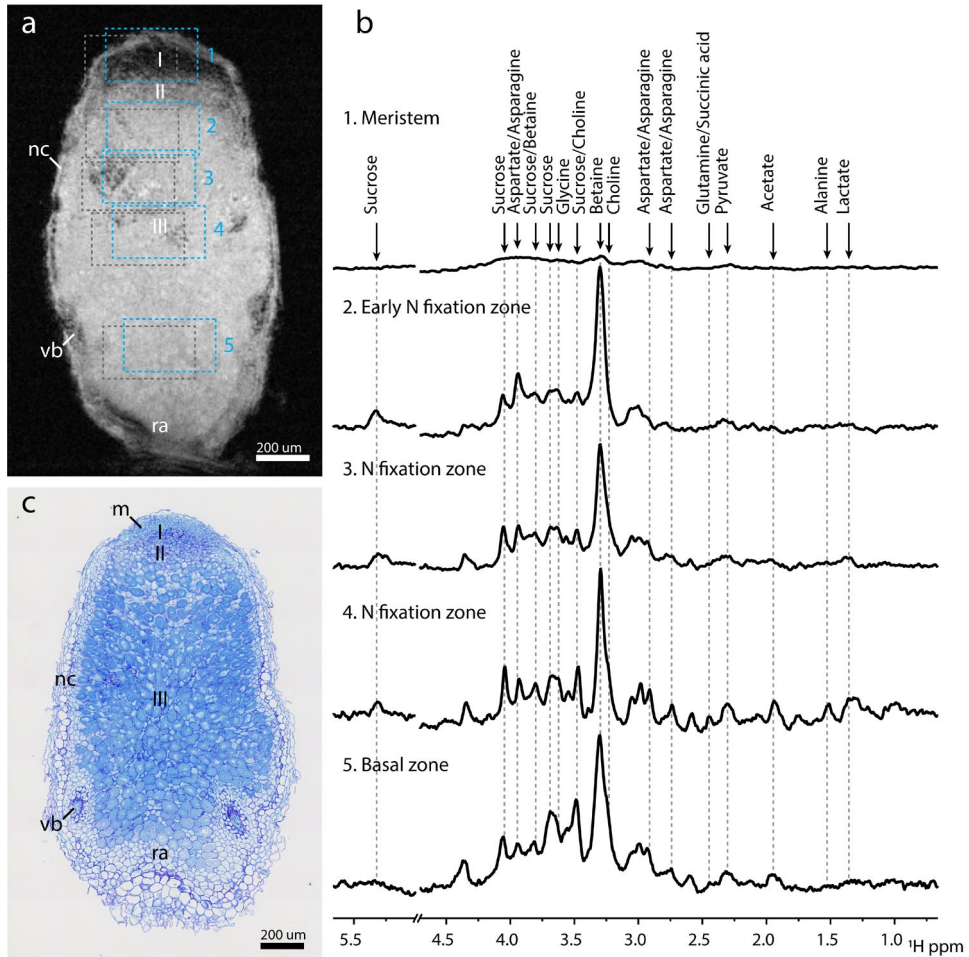


Figure 4.3 Localised spectroscopy in *in-situ* root nodule reveals sugar differences in pre- and post-granule onset regions. **(A)** FLASH reference with PRESS Region of Interest (ROI), numbered one through five. Roman numerals indicate nodule zones: Meristem (I), Infection zone (II), Nitrogen fixation zone (III). Grey dotted boxes show ROI shift of Betaine due to Chemical Shift Displacement Error (CDSE). Pulse excitation was centred around 4.0 ppm. **(B)** Co-registration of Optical Microscopy confirms the presence of bacteroids in the active nitrogen fixation region. Toluidine blue staining 10x magnification. **(c)** PRESS spectra captured from Regions of Interest (ROI) shown in Fig. 3A, for Nodule Meristem (1), (early) Nitrogen fixation zone (2), Nitrogen fixation zone (3 & 4), Basal region (5). PRESS voxel sizes were $200 \times 350 \times 350 \mu\text{m}^3$. Spectra were aligned to the betaine peak at 3.3 ppm. Water peak region at 4.7 ppm has been omitted for ease of viewing. Intensities of the spectra have not been normalised, reflecting the strength of the signal recorded. This means that for the meristem little information can be discerned. Line broadening 10 Hz. Abbreviations: nc, nodule cortex; vb, vascular bundle; m, meristem; ra, root attachment area. Scale bars 200 μm .

4.3.2 MR SPECTROSCOPY REVEALED THE SPATIAL DISTRIBUTION OF NODULE METABOLITES

Since the root nodule is an active plant organ whose primary function is to fix nitrogen and to facilitate nutrient exchange, SNF-linked metabolite distribution over the various nodule zones is of interest. According to earlier literature, a nodule may be divided into four zones, based on SNF activity: (I) meristem zone, (II) infection zone, (III) nitrogen fixation zone, and (IV) senescence zone, the latter observed mostly in older nodules. In the infection zone, bacteroids accumulate and mature, while in the fixation zone active SNF takes place (Dupont *et al.*, 2012). Localised Magnetic Resonance Spectroscopy (MRS) was utilised to distinguish metabolites, based on their frequency differences (chemical shift) and location. Localised MR spectra were recorded from five different regions of interest (ROI), localised in the four different zones in an intact freshly excised root nodule, *in situ* (Fig. 4.3A). The presence of bacteroids was verified with optical microscopy after the MRI measurements (Fig. 4.3C). The first ROI was placed on the meristem (ROI 1) in Fig. 4.3B, where little MR signal was recorded, possibly due to the small volumes of individual cells which may cause T_2 -shortening, reducing the SNR. The second voxel (ROI 2) was placed in the infection/early fixation zone. The third (ROI 3) and fourth voxels (ROI 4) were placed in the largest zone: the active nitrogen fixation zone. The last voxel (ROI 5) was placed in the basal region of the root nodule.

The spectra obtained from ROIs 2-5 showed the presence of several metabolites including various amino acids, sugars, choline and betaine. Betaine was present in a high amount in all four ROIs (Fig. 4.3B). The signal from sucrose and asparagine/aspartate was also clearly detected. Assignment of various metabolites was confirmed with solution-state NMR performed on an extract obtained from the root nodules, using two-dimensional ^1H - ^1H correlation spectroscopy (2D-COSY) (see Supplementary Fig. 4.S3). It should be noted that, due to the chemical shift displacement error, which increases with magnetic field strength, the regions of interest chosen for the localised spectra were shifted slightly for each metabolite, as the spatial selection relied on the frequency of the metabolite relative to the chosen centre frequency of 4.0 ppm (van der Graaf, 2010). This shift meant that the indicated regions of interest in Fig. 3a were correct for signals at $\delta = 4$ ppm, while for example the strongest signal (at $\delta = 3.3$ ppm) originated from an ROI which was shifted by 35 μm along x, 97 μm along y and 97 μm along z (see the dotted region of interest in Fig. 4.3A).

The strongest peak observed in all spectra was attributed to a resonance of glycine betaine ($\delta = 3.3$ ppm) (Fig. 4.3B). A complementary spectroscopic imaging method, Chemical shift imaging (CSI) confirmed the presence of betaine throughout the nodule (Supplementary Fig. 4.S4). Betaine serves as an important osmoprotectant produced by *S. meliloti* (Smith *et al.*, 1988). Furthermore choline - a precursor to glycine betaine in both plants and bacteria - was visible in ROI 3 as a shoulder on the low frequency side of the betaine peak, and was even more pronounced in ROIs 4 and 5 (Ashraf and Foolad, 2007). In uninfected root tissue, choline, but not glycine betaine was detected as seen in solution NMR measurements (Supplementary Fig. 4.S5).

In root nodule, another strong peak from asparagine and aspartate was seen primarily in ROIs 2-5 (Fig. 4.3B). Notably, glutamine - though present in ROI 4 - appeared much lower in concentration in all ROIs than asparagine, suggesting a smaller pool or higher turnover rate in the nitrogen assimilation process.

Intermediates from the tricarboxylic acid cycle such as acetate ($\delta = 1.9$ ppm), pyruvate ($\delta = 2.3$ ppm) and succinic acid ($\delta = 2.4$ ppm) were also seen in the nitrogen fixation zone (region 3 & 4). Lactate ($\delta = 1.3$ ppm) was visible only in the active nitrogen fixation zone (region 3 & 4). Alanine ($\delta = 1.5$ ppm) has been suggested by metabolic network modelling to be the primary metabolite for the uptake of assimilated nitrogen by plants (Pfau *et al.*, 2016). However, our results indicated that the pool of alanine was small compared to asparagine.

Sucrose, the most important resource for growth and root nodule activity, was present in all regions with a concentration gradient towards the basal area, starting with the early fixation zone (Fig. 4.3B, ROI 2). Several peaks were assigned to sucrose (*e.g.*, $\delta = 5.3$ ppm), with some overlap with choline ($\delta = 3.5$ ppm) and fructose ($\delta = 3.8$ ppm). In the basal region (region 5), the sucrose peak at $\delta = 5.3$ ppm was hard to distinguish, possibly due to baseline distortions. Overall, the metabolic pattern exhibited in regions 3-4 appeared to be maintained in the basal region (region 5), though attenuated and with a larger linewidth, possibly reflecting a more disorganised structure of the tissue. At the same time active SNF still appeared to take place, indicating this region is not (yet) the senescent zone IV in which the activity would be reduced.

4.3.3 MICROSCOPIC DETECTION OF STARCH CORRELATES WITH MR BASED SUCROSE PROFILE

Starch is a carbohydrate storage polymer that is unique to plants. Indeterminate root nodules are known to form amyloplasts in certain cells, detectable by optical microscopy, especially in the youngest cells of the nitrogen-fixation zone of an actively growing nodule (López *et al.*, 2008). Accumulation of amyloplasts has also been found in cells located behind the division zone in shoot meristems (Jordy, 2004). Zeeman *et al.* speculated that this might be caused by 'a temporary imbalance between carbon import and utilisation as cells move from division to expansion and differentiation' (Zeeman, Kossmann and Smith, 2010). If correct, this marker for high metabolic activity should be reflected in changes to the metabolic profile of bacteroid containing cells. Therefore, I aimed to detect pre- and post-starch granule formation zones in an intact root nodule, and correlate them with sugar metabolites as measured by localised spectroscopy.

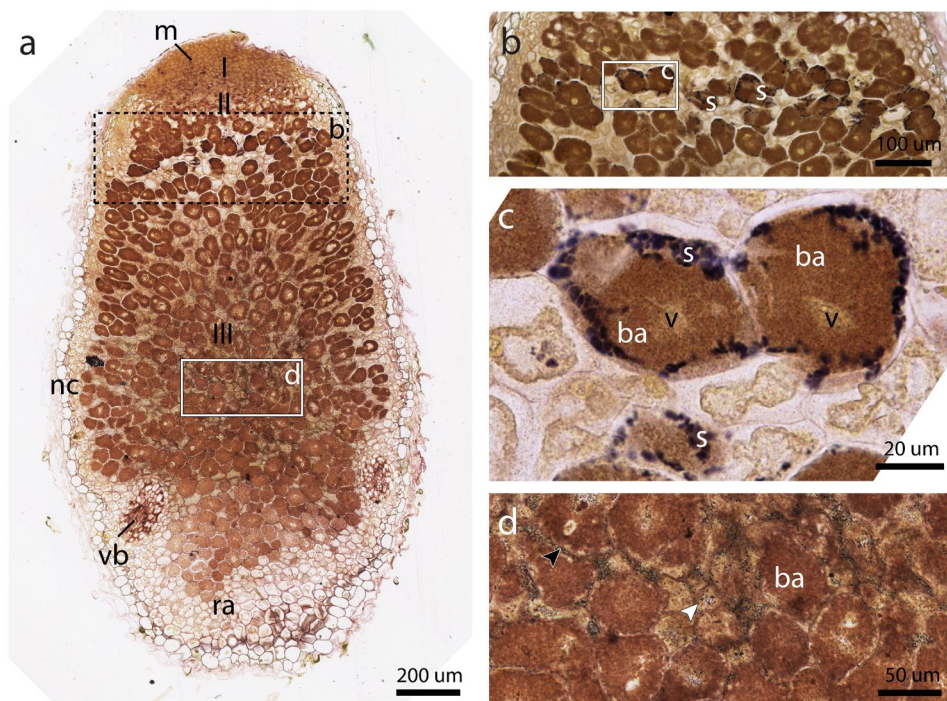


Figure 4.4 Optical microscopy of root nodules reveals multiple starch distribution patterns. Coupes were stained with both Lugol's solution and Toluidine blue. (A) A section immediately adjacent to that of Fig. 3A reveals starch distribution throughout the nodule. 10x magnification, scale bar 200 μ m. A black dotted rectangle indicates the zone shown in (B) on an alternate section. A white rectangle indicates an enlargement seen in (D). 10x magnification, zoomed; scale bar 50 μ m. (B) At the first layer of cells in zone III, starch is present as a band (s). A white rectangle indicates the area shown in (C). Alternate section, 20x Magnification, scale bar 100 μ m. (C) Starch granules (s) are located near the periphery of infected cells. The vacuole (v) reappears in the fixation zone after disorganisation in the infection zone. (B). 40x Magnification, scale bar 20 μ m. (D) A fine distribution of starch granules can be seen in Zone III contained in nitrogen fixing cells (black arrowhead) and uninfected cells (white arrowhead). Abbreviations: ra, root attachment site; nc, nodule cortex; vb, vascular bundles; m, meristem; s, starch granules; v, vacuole; ba, bacteroids.

Lugol's solution was used to stain amyloplasts (Fig. 4.4A) on an optical section immediately adjacent to that of Fig. 4.3B, to relate the distribution of sucrose and other metabolites to starch. Starch distribution was seen as dark spots, concentrated in a band located in the first layer of the nitrogen fixation zone as well as in smaller amyloplasts throughout the fixation zone (Fig. 4.4B and 4.4D). In the nitrogen fixation zone, amyloplasts were small and dispersed over infected cells (black arrowhead) as well as uninfected interstitial cells (white arrowhead)(Fig. 4.4D). In contrast, the cells present in the first layer on the border of the infection zone (II) and fixation zone (III) exhibited a different distribution of starch (Fig. 4.4B). At higher magnification, large amyloplasts were visible at the periphery of the infected cells, which were almost completely filled with bacteroids (Fig. 4.4C). Thus, sucrose and starch appear to co-localise with SNF

activity. Taken together, starch distribution patterns could provide a possible biomarker for changes in cellular metabolism during nodule development.

4.4 DISCUSSION

This paper showed the feasibility of attaining cellular resolution $7 \times 7 \times 7 \mu\text{m}^3$ in *M. truncatula* root nodules over a relatively large volume $1.8 \times 1.4 \times 1.4 \text{ mm}^3$, opening up new applications for MR microscopy in plant studies (Fig. 4.2A). Individual cells, especially young and mature bacteroids containing cells, were resolved with high contrast, exhibiting a unique 'grey ring' pattern where *S. meliloti* cells accumulated near the cell walls around a central vacuole. Smaller cells, such as present in the meristem, were too small to be resolved individually. The low intensity of the meristem may also reflect a short T_2 relaxation time relative to other structures. Looking towards *in situ* imaging, nodules suffered from lower image contrast and SNR (Fig. 4.2C). A possible reason for the lower image quality may be the abundance of air spaces due to the lack of fixation and vacuum treatment. More quantitative measurements are required to determine the causes of reduced SNR and contrast.

Although the hypo-intense regions caused by small and large air pockets can be detrimental to overall image quality (Supplementary Fig. 4.S2), they also contain useful information. Air pockets in root nodules of *M. truncatula* are of particular interest, because of the delicate balance of oxygen levels required for the proper functioning of the nitrogen fixation. The pockets may thus have a regulatory role, as nitrogenase is paradoxically both dependent on oxygen for carrying out its function but can also be poisoned by oxygen under high concentrations (Lundquist, 2000). Previous MR investigations into root nodules of soybean (*Glycine max*) have focused on investigating the oxygen diffusion barrier, believed to regulate nodule functioning (Brown *et al.*, 1997; Chudek *et al.*, 1997). Furthermore, *Glycine max* root nodules are known to be sensitive to changes in gas perfusion (Macfall *et al.*, 1992). Thus, MRM could be a useful tool to study oxygen regulation mechanisms, due to its ability to detect air spaces through enhanced signal loss.

PRESS localised spectroscopy results show that betaine was distributed throughout the nodule (Fig. 4.3C). The relatively high concentration of betaine could be due to maintaining a high degree of osmoregulation, an indicator of stress, or both (Ashraf and Foolad, 2007). High asparagine levels seen especially in the nitrogen fixation zone indicate significant SNF activity. The presence of asparagine, as well as betaine, was also confirmed by high-resolution 2D correlation spectroscopy on extracts prepared from nodules (Supplementary Fig. 4.S3). Asparagine, together with glutamine, is known to be a major export product of assimilated nitrogen (Sulieman and Tran, 2013). Our results show that asparagine accumulates mostly in active SNF areas (regions 3-5). As such, asparagine was absent in normal root tissue (Supplementary Fig. 4.S5).

Metabolite levels of betaine and asparagine might therefore potentially provide useful markers for stress and SNF activity, respectively. The sucrose concentration gradient,

decreasing from the basal to the apical end of the nodule, may reflect either consumption rates or limitations in transport through the nodule. In conjunction, starch granules appeared to be localised to two specific regions, a band in the transition from infection to fixation zone, and the fixation zone (III) itself (Fig. 4.4). The band seemed to provide support for Zeeman's prediction of amyloplast formation due to a 'temporary metabolic imbalance', though it is located at some distance from the meristem (Fig. 4.4B) (Zeeman, Kossmann and Smith, 2010). As for the fixation zone, it has been observed in the literature that starch amyloplast accumulation in uninfected cells is related to the expression of SNF-related (*nif*) genes (Fig. 4.4D) (Hirsch, Bang and Ausubel, 1983; Hirsch and Smith, 1987). Considering that sucrose was present throughout the nodule, albeit in a diminishing concentration gradient, it is likely that cell-specific metabolism and environmental conditions determine whether and in what quantity starch amyloplasts are formed (Prell and Poole, 2006; Dupont *et al.*, 2012). For instance, sucrose must be metabolised by the host cell in order to provide carboxylic acids that are preferentially taken up by the bacteroids (Yang, Udvardi and Day, 1990; Hohnjec *et al.*, 2007). The observed distribution in size and location of amyloplasts could be affected in this way as well.

As an explorative study of ultra-high field MRM for plant imaging, several recommendations might be of interest to the reader. Sample handling and preparation are essential for high-resolution imaging. More generally, special attention needs to be given to managing sample susceptibility at ultra-high fields, since the inclusion of even small air bubbles in the vicinity of samples can distort image quality significantly, as well as cause line broadening in localised spectroscopic experiments. Another source of sample susceptibility could be the presence of iron in the form of leghaemoglobin, which regulates oxygen levels. In bacteroids of mature nodules, up to 25% of the total soluble protein consists of leghaemoglobin (Nap and Bisseling, 1990). The high concentrations of this iron-heme containing protein might have implications for the values of longitudinal relaxation (T_1) and transverse relaxation (T_2) and their interpretation, as these properties may depend strongly on the physiological state of the root nodule when measured. Thus, with further characterisation, susceptibility-weighted imaging may provide a useful method for evaluating the physiological state of root nodules.

Rather than a higher resolution per se, the future applications for MRM at ultrahigh field for – *in vivo* – imaging are also promising, including longitudinal studies. However, to achieve longitudinal imaging novel coil-insert designs are required with the necessary life support systems. The solenoid coil type used here could be modified to allow for a relatively simple setup that would support whole plants. Vertical-bore MRI systems allow top access to supply light and fresh medium. Additionally, controlled nutrient supply would be desirable, *e.g.* a continuous medium perfusion setup (Flint *et al.*, 2017). Overall, an imaging setup optimised for longitudinal studies would allow testing of stress conditions for host-symbionts, as spectroscopic information can be acquired non-destructively, for example every 30 minutes.

In conclusion, our results demonstrate that MRM at ultra-high fields in conjunction with microcoils provides a promising technique to determine physiology and metabolic profiling non-invasively in plant root nodules. Further research and development on coil design are required to exploit this application to its full potential.

4.5 METHODS

4.5.1 GROWING CONDITIONS *MEDICAGO TRUNCATULA*

Medicago truncatula accession R108 was grown in perlite saturated with Färhaeus medium without nitrate in a growth chamber at a temperature of 21 °C and 16/8 h light/dark cycle (Limpens *et al.*, 2004). Plants were inoculated with *Sinorhizobium meliloti* Rm41 ($OD_{600} = 0.1$, 2 mL per plant) (Weidner *et al.*, 2013). Root nodules were collected for analysis 21 days after inoculation. Perlite was used as a soil replacement to facilitate the extraction of intact root systems.

4.5.2 SAMPLE PREPARATION

For MRI measurements, a single *M. truncatula* plant was carefully extracted from the perlite substrate. Healthy nodules that exhibited a light-pinkish colour were selected and used for MRI imaging. For *in situ* MRI measurements, a nodule was excised and was directly used for measurements. For *ex vivo* MRI and optical microscopy, root nodules were fixed with paraformaldehyde (4% w/v) and glutaraldehyde (5% v/v) in 50 mM phosphate buffer (pH 7.2) at 4 °C overnight, followed by vacuum treatment for 30 minutes. Samples were then stored at a temperature of 4 °C until use.

Using an SZ40 stereomicroscope (Olympus, Tokyo, Japan), individual nodules were selected that could fit within capillaries with an inner diameter of $(1050 \pm 50) \mu\text{m}$ (Hilgenberg GmbH, Malsfeld, Germany). To avoid artefacts during MRI acquisition from air bubbles both within and outside the sample, Perfluorodecalin (PFD) was used to submerge the sample. PFD was chosen because it has several advantageous properties, making it suitable as an infiltration agent. Importantly, it is a non-toxic compound, capable of dissolving both CO_2 and O_2 (Littlejohn and Love, 2012). Furthermore, PFD exhibits a low surface tension ($1.9 \times 10^{-2} \text{ N m}^{-1}$), below the limit for stomatal penetration ($2.5\text{-}3.5 \times 10^{-2} \text{ N m}^{-1}$) in *Arabidopsis thaliana* leaves (Schönherr and Bukovac, 1972; Littlejohn and Love, 2012), making it useful for reducing air bubbles within the sample without entering the cells. Care was taken to minimise the required amount of PFD as it is known to function as a potent greenhouse gas (Tsai, 2011).

During insertion of the nodule in the centre position of the capillary, both nodule and capillary were kept submerged in PFD. Capillary wax (Hampton Research, California, USA) was then applied to both ends of the open capillary with a MaxWax Pen (Hampton Research, California, USA). Considerable effort was spent on minimising the inclusion of air bubbles within the capillary.

For solution NMR measurements, metabolites were extracted from root and root nodules as described in detail by Kim *et al.* (2010) (Kim, Choi and Verpoorte, 2010). Key

steps included manual excision of root and root nodule tissue and consequent crushing with mortar and pestle in the presence of liquid nitrogen. Samples were then freeze-dried and lastly extracted with an extraction buffer consisting of 50% (v/v) deuterated methanol and 50% D₂O phosphate buffer (pH 6) (Kim, Choi and Verpoorte, 2010).

4.5.3 MRI MEASUREMENTS

A custom solenoid microcoil was built in order to achieve the necessary sensitivity required for high-resolution imaging. For the microcoil assembly, a glass-fibre circuit board was used as a base to hold all the necessary components in place. The radiofrequency (RF) coil consisted of coated 28 AWG ($\varnothing$ 0.4mm) copper wire, wound into a solenoid with six windings around a glass capillary with an outer diameter of (1500 ± 50) μ m. A fixed 1.5 picoFarad (pF) capacitor was placed in series with the RF coil. To complete the resonant circuit, a single 1.5-6 pF variable capacitor was placed parallel to the RF coil and 1.5 pF capacitor, which allowed for fine-tuning the resonant frequency to 950 MHz. The circuit board was then attached to a Micro5 probe (Bruker Biospin, Ettlingen, Germany) compatible circuit holder.

MRI measurements were performed on a Bruker 22.3 T (950 MHz) spectrometer (Bruker Biospin, Ettlingen, Germany), at the uNMR-nl national facility (Utrecht, The Netherlands). The magnet had a vertical bore of 54 mm in diameter and was connected to an Avance III HD console. A Bruker Micro5 probe with exchangeable RF inserts allowed operation of the 1500 μ m custom-built solenoid resonator. The Micro5 probe contained a built-in 48 mT m⁻¹ A⁻¹ (2.88 T m⁻¹ at 60 A) triple axial gradient system coupled to GREAT 60 A amplifiers and cooled with a BCU 20 water cooler. Control of spectrometer operations was performed with Paravision 6.0.1 and Topspin 3.1PV running on a CentOS workstation. Sample temperature was maintained at (298 ± 1) K using a BCU II cooler.

High-resolution 3D Fast Low Angle Shot (FLASH) images were acquired with the following parameters: matrix size $256 \times 192 \times 192$; read direction along the largest matrix direction; the field of view was $1.8 \times 1.4 \times 1.4$ mm³. Resolution $7 \times 7 \times 7$ μ m³ isotropic, number of averages 28, repetition time 120 ms, flip angle 30° and echo time 2.9 ms. Receiver bandwidth was set to 100 kHz. To allow for co-registration with coupes from optical microscopy, the JIVE tool was utilised to generate oblique slices.

A multiple gradient echo (MGE) sequence was also utilised to evaluate the presence of air bubbles and its influence on the image quality. A total of 32 gradient recalled echoes were acquired; initial echo was 2.7 ms, further echoes were spaced 2.8 ms apart resulting in echo times of 2.7, 5.5, 8.3, ..., 89.5 ms. Matrix size was $128 \times 64 \times 64$; Read direction along the largest matrix direction; field of view was (1.8×1.4) mm². Slice thickness was 1.4 mm; resolution $19 \times 19 \times 19$ μ m³. Number of averages was 24. Repetition time was 120 ms; flip angle 5°.

4.5.3.1 LOCALISED SPECTROSCOPY

Point Resolved Spectroscopy (PRESS) was used for localised magnetic resonance spectroscopy (MRS) on root nodules (Bottomley, 1984, 1987). PRESS employs 90°-180°-

180° orthogonal pulses with concurrent slice selective gradients. The PRESS sequence was preceded by a VAPOR (Variable Pulse Power and Optimized Relaxation Delays) sequence for global water suppression, which consists of seven variable power RF pulses with an optimised relaxation delay (Tkáč *et al.*, 1999). Appropriate volumes of interest (voxel) for PRESS were selected from reference FLASH images. Correct positioning of the voxel was also verified afterwards by high-resolution FLASH. Five individual MRS voxel (200x350x350 μm^3) were localised covering various zones in the nodule. The local field homogeneity was optimised over the voxel by MAPSHIM. The field homogeneity was further improved by manual shimming of up to second order shims (Z-Z²-Z-X-Y-Z-Z²-Z-XY-XZ-YZ-Z). The field homogeneity resulted in a water line width of 10 Hz. For PRESS measurement, repetition time was set to 1000 ms, echo time was 7.2 ms, and 2048 averages were acquired for a total acquisition time of 34 m 8 s. Spectra were manually phased and automatically baseline corrected with a 3rd or 6th order Bernstein-polynomial fit. Chemical shift alignment was adjusted to the largest peak (3.3 ppm).

4.5.3.2 CHEMICAL SHIFT IMAGING

Chemical Shift Imaging (CSI) was used to acquire a multi-voxel spatial map of metabolic distribution. A large volume of interest was selected using a PRESS approach, consisting of three mutually orthogonal slice selective pulses. Then phase encoding occurred in two directions by applying incremental pulse gradients in each direction, resulting in a matrix of spectroscopic spin echoes.

The following basic parameters were used: Repetition time = 760 ms; echo time = 3 ms; Matrix = 24 × 16; FOV = 1.8 × 1.2 mm²; slice thickness = 400 μm ; resolution = 150 × 150 × 400 μm^3 ; number of averages = 1024. Spectra were acquired with 2048 points; dwell time 26.4 μs ; spectral width 19.93 ppm (18,939 Hz). Magnetic field homogeneity in the selected volume was optimized by shimming the water resonance. A VAPOR suppression scheme was applied for efficient water signal saturation before CSI acquisition.

Reference FLASH images were acquired with the following parameters: matrix size was 96x64; Read direction along the largest matrix direction; field of view was 1.8 × 1.2 mm². Slice thickness was 0.1 mm; resolution 19 × 19 × 100 μm^3 isotropic. Number of slices 4; number of averages 128; acquisition time 28 m 40 s. Repetition time was 210 ms; flip angle 30° and echo time 3.26 ms. Receiver bandwidth was set to 50 kHz.

For the processing of the CSI data, the integration of selected signal of specific metabolite areas in magnitude mode was overlaid on reference FLASH images using the Bruker CSI Visualisation Tool.

4.5.4 NMR MEASUREMENTS

NMR measurements were performed on a Bruker 20.0 T (850 MHz) spectrometer with a vertical bore 54 mm in diameter connected to an Avance III HD console. A triple-tuned broadband cryoprobe was used. Topspin 3.2 was used to control the spectrometer and for processing of the acquired data. The sample temperature was maintained at 298K.

¹H NMR spectra were collected using a zgpr30 sequence with pre-saturation to suppress water efficiently. Data was acquired into 65k points; dwell time 29.3 μ s, spectral width 20 ppm or 17,045 Hz; number of averages, 512; dummy scans 4; pre-scan delay 10 μ s. 2D homonuclear ¹H-¹H experiments were performed using chemical shift correlated spectroscopic sequence (COSY). The following parameters were used: 3400 points in the direct dimension, 400 points in the indirect dimension in Digital Quadrature Mode. Both dimensions zero-filled to 4096 points. Number of averages, 96; dummy scans, 16; Spectral width 12.0 ppm, 10,000 Hz; Temperature 298K.

Assignments were cross-checked against metabolite databases and fitting was performed using Chenomx deconvolution software (Chenomx, Edmonton, Canada)(Cui *et al.*, 2008; Wishart *et al.*, 2009). 1D reference spectra were acquired with parameters as follows: data was acquired into 65k points; dwell time 41.6 μ s, spectral width 20 ppm or 12,019 Hz; number of averages, 64; dummy scans 4; pre-scan delay 10 μ s. COSY reference spectra were acquired with the following parameters: 4096 points in the direct dimension, 256 points in the indirect dimension in Digital Quadrature Mode. Indirect dimension zero-filled to 4096 points. Number of averages, 4; dummy scans, 16; Spectral width 12.3 ppm, 7200 Hz; Temperature 298 K.

All experimental data were acquired and processed using Paravision 6.0.1 (Bruker Biospin, Ettlingen, Germany) and Topspin 3.1 running on Linux. The default bi-cubic interpolation was applied to enhance the details of the image prior to exporting images. Figures were prepared in Adobe Photoshop CC 2019 and Adobe Illustrator CC 2019 (Adobe Systems Incorporated, Mountain View, California, USA). Stacked spectra were produced in MestReNova (MestreLab Research S.L., Santiago de Compostela, Spain).

4.5.5 LIGHT MICROSCOPY

After MR imaging, the sample material was dehydrated in an ethanol series and subsequently embedded in Technovit 7100 resin (Heraeus Kulzer, Hanau, Germany) according to the manufacturer's protocol. Sections (5 μ m) were made by using a microtome (Reichert-Jung, Leica Microsystems, Netherlands), stained with 0.05% Toluidine blue O for 1 min and additionally with Lugol's iodine solution for a few seconds (if applicable). Sections were analysed by using a DM5500B microscope equipped with a DFC425C camera (Leica Microsystems, Wetzlar, Germany).

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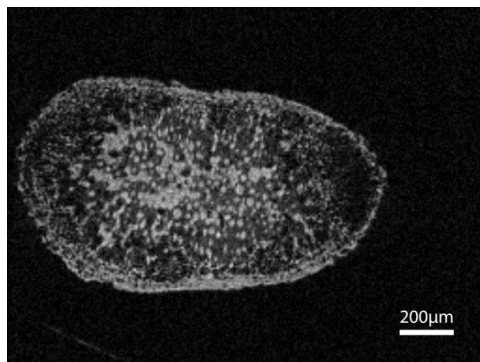
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4.7 ACKNOWLEDGEMENTS

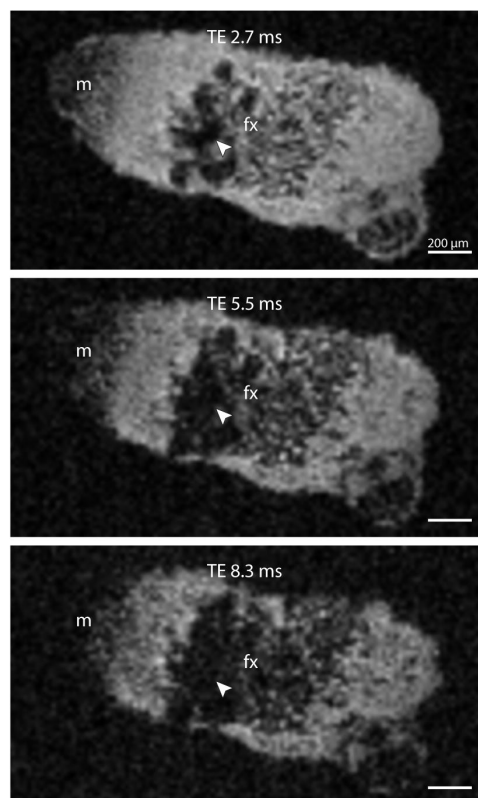
Experiments at the 950MHz instrument were supported by uNMR-NL, an NWO-funded National Roadmap Large-Scale Facility of the Netherlands (project 184.032.207). R.S. was supported by the BioSolarCells consortium project U2.3. J.R.K. was supported by the NWO-funded graduate school Netherlands' Magnetic Resonance Research School (NMARRS) graduate school [022.005.029]. D.S. was supported by China Scholarship Council (201306040120). A.G.W. was partially supported by European Research Grant 670629 NOMA MRI. Klaartje Houben, Marie Renault and Johan van der Zwan are thanked for technical support at the uNMR-NL facility. Further thanks go out to Volker Lehmann, Henny Janssen and Pieter de Waard for help in troubleshooting.

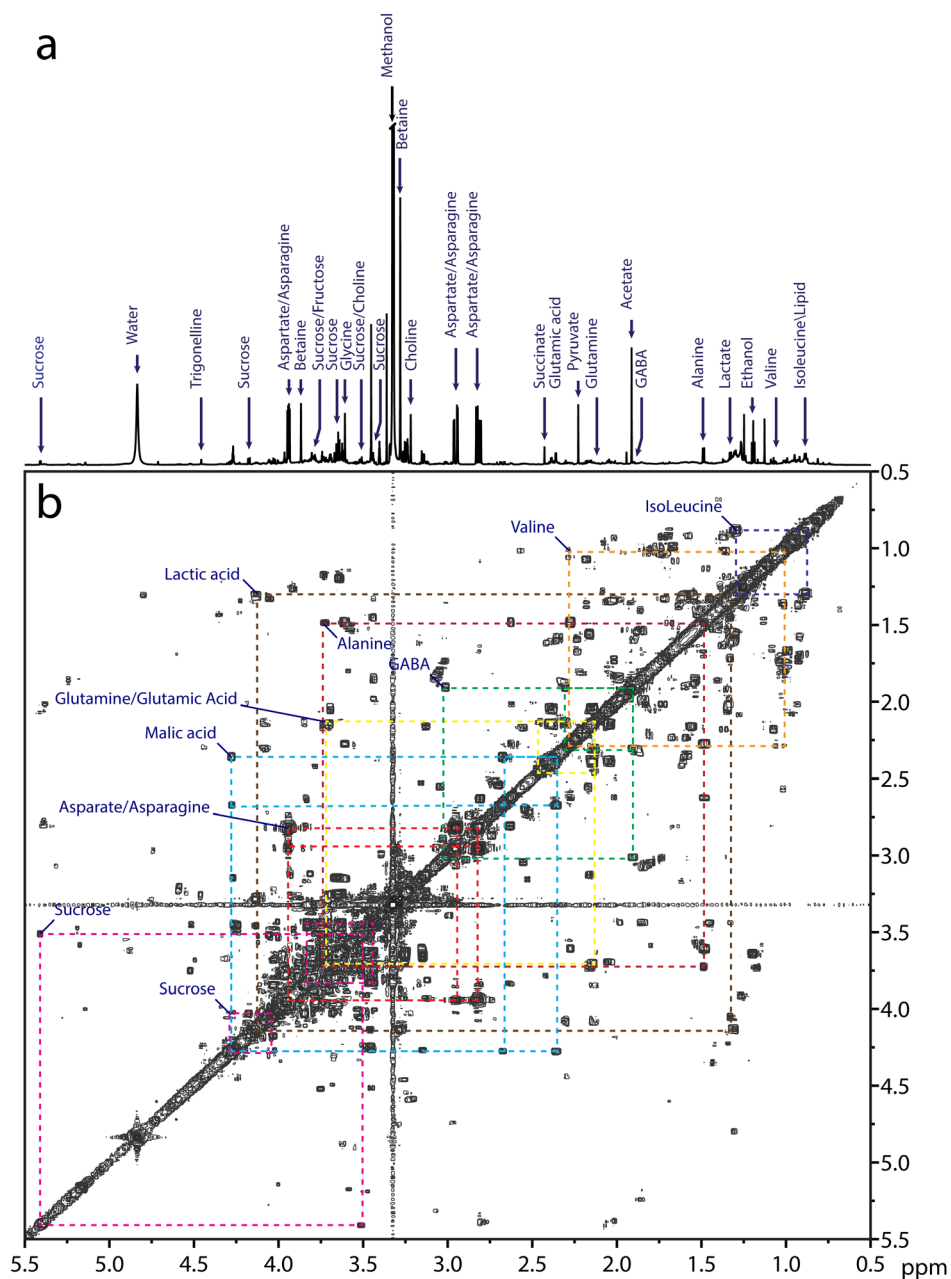
4.8 SUPPLEMENTARY INFORMATION

4.S1 Video High-resolution 3D FLASH video of fixed and vacuum treated root nodule. Sliced along original matrix orientation. Aspect ratio 4:3, video resolution 1036x768 pixels, 25 frames per second. Duration 6.4 s, 160 frames. Scale bar 200 μm .

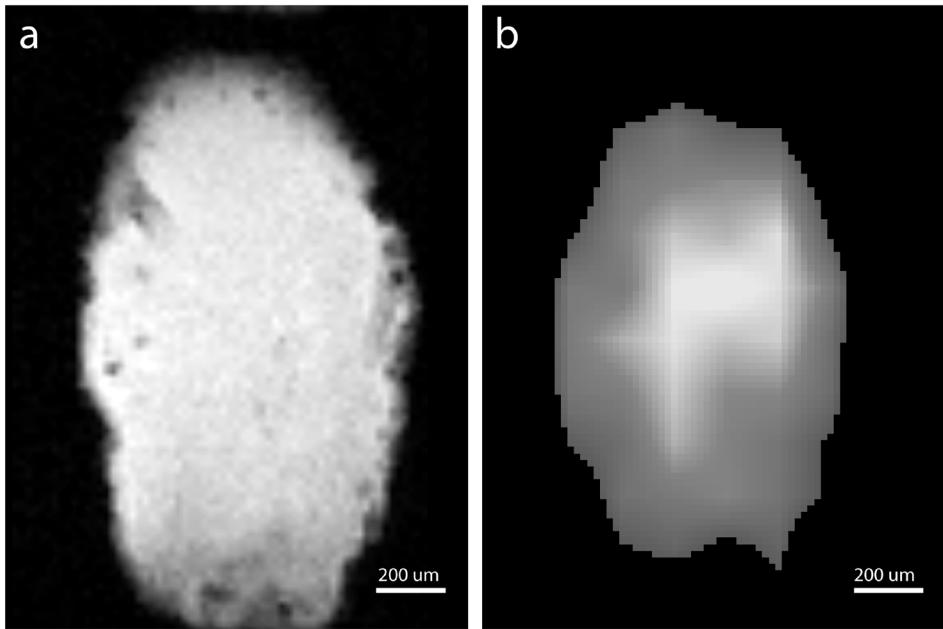


4.S2 Fig. T₂-weighted images reveals susceptibility artefacts caused by air pockets. An MGE sequence was used to acquire scans with 2.7, 5.5, 8.3 ms echo time on a fixed but not vacuum treated nodule, showing a progressive loss of signal as a function of echo time. Nodule Apical-Basal orientation is left-to-right. Dark areas are indicative of air pockets that dephase signal through increased local magnetic susceptibility differences (arrow). Notably, air pockets are variable in size with smaller pockets seen in the basal direction. The meristem and nitrogen fixation area are particularly affected by the signal loss, though the meristem signal loss is likely due to short T₂, not susceptibility. Abbreviations: fx, fixation zone; m, meristem. Scale bar 200 μm .

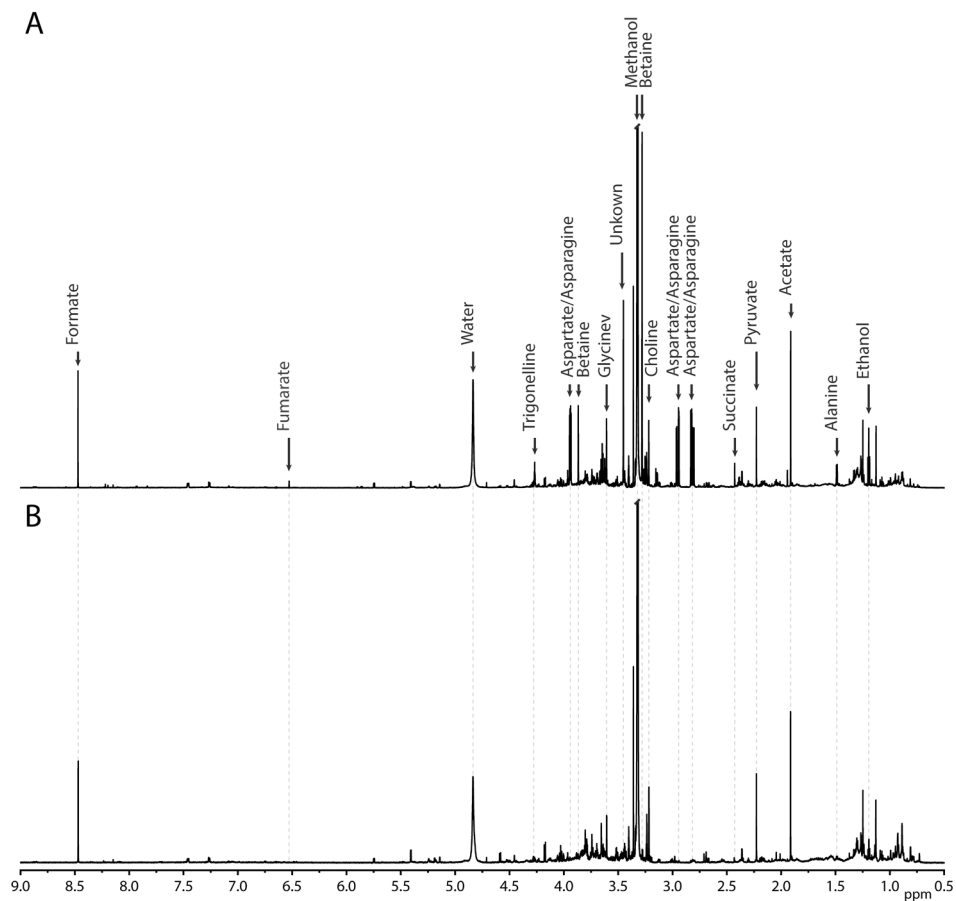




4.53 Fig. Homo-nuclear ^1H correlation spectroscopy (COSY) of root nodule. **(A)** 1D NMR from 0.5 to 5.5 ppm with the most abundant metabolites assigned. Solvent Methanol/ D_2O , water suppressed. GABA = γ -aminobutyric acid. **(B)** COSY was used to verify assignments of *in situ* PRESS results. COSY assignments for the most relevant metabolites are indicated with coloured boxes. Range 0.5 to 5.5 ppm, water suppressed.



4.54 Fig. Chemical Shift imaging showing betaine localisation. Nodule Apical-Basal orientation is top to bottom. **(A)** MSME reference image. Note that the CSI volume of interest is larger than the nodule itself. Thus, the distribution of metabolites may be skewed. **(B)** Heat map generated from betaine peak (integration bandwidth 300 Hz).



4.55 Fig. A representative solution state ^1H NMR spectrum of root and nodule tissue, revealing differences in several major metabolites. Spectra acquired at 850 MHz. **(A)** Nodule tissue **(B)** Root tissue.

5 GENERAL DISCUSSION AND OUTLOOK

This thesis explored applications of ultra-high field MRI and microcoils to imaging algae and plants. In particular, the unique perspective of MRI in terms of localised spectroscopy and diffusion measurements was brought to the investigation of *Botryococcus braunii*. Furthermore, microcoils were developed, characterised and used to study *Medicago truncatula*.

5.1 OIL LOCALISATION IN *B. BRAUNII* AND BEYOND

The hydrocarbons and oil found in strains of *Botryococcus braunii* strains are a defining feature for the species, as they are implicated in its organisational structure (Weiss *et al.*, 2012; Tanoi, Kawachi and Watanabe, 2014). While there is detailed knowledge of *B. braunii* physiology and anatomy on a cellular level, such information is presently lacking for colony sizes studied here (Suzuki *et al.*, 2013; Uno *et al.*, 2015). In Chapter 2, it was shown that oil localisation is correlated with colony structure, more specifically, colonies exhibiting a hydrocarbon distribution concentrated in two concentric shells. The nature of the two shells and importantly, the causes for their development, warrant further investigation. The distribution of oil in two shells also raises questions on the mobility of oil and water within colonies. Especially the implications for the colony physiology are of interest, since this could relate to growth mechanisms and growth speed.

Two distinct colony types could be distinguished by MR chemical shift (selective) imaging, with the larger type exhibited water channels. Diffusion measurements revealed Apparent Diffusion Coefficient (ADC) values of water linked to the presence of oil and hydrocarbons. However, questions remain concerning the possible development processes through which the two different colony types could have formed. A range of hypotheses is possible, including colony fusion and daughter colony formation. In order to study these hypotheses, longitudinal studies under varying environmental conditions are required to establish the mode of formation and whether these types exist from an early stage or developed at a later stage. MRI would be appropriate for such studies as it is highly suited to longitudinal studies of intact, *in-vivo* organisms due to its non-invasive nature (Musse *et al.*, 2009; Van As, Scheenen and Vergeldt, 2009; Van As and van Duynhoven, 2013). Potentially, culturing could even be done within standard NMR tubes, if outfitted with sparging, a light source and regular medium changes.

5.2 DIFFUSION WEIGHTED CHEMICAL SHIFT IMAGING

While in chapter 2, CSSI and CSI were able to localise the oils and hydrocarbons present in *B. braunii*, the presence of water channels raises questions concerning the diffusion of nutrients and oils inside the colonies. Especially the interplay between oil, water and metabolite diffusion is of interest. Since diffusion imaging (DWI) has difficulty distinguishing multiple compounds, as it relies on fitting the combined signal decay, new methodology is required to collect localised information on multiple compounds.

Diffusion Weighted Chemical Shift Imaging (DW-CSI) could potentially satisfy these requirements (Ercan *et al.*, 2015). The DW-CSI sequence is particularly elegant because it combines two of the most attractive capabilities of MRI in a relatively simple manner: localised spectroscopy and diffusion-weighted imaging. While CSI is capable of differentiating multiple molecular species, the addition of a DW module captures information on their respective local diffusive environment, such as vesicle or cell size.

Preliminary results for the DW-CSI have been obtained on a phantom at 17.6T, see Fig. 5.1A. Heatmaps indicate the signal present for a selected resonance, which attenuates as diffusion encoding is increased (Fig. 5.1 B, C, and D).

A series of DW-CSI spectra with increasing diffusion encoding may be stacked in a white-wash plot to visualise the signal decay curves, see Fig. 5.2.

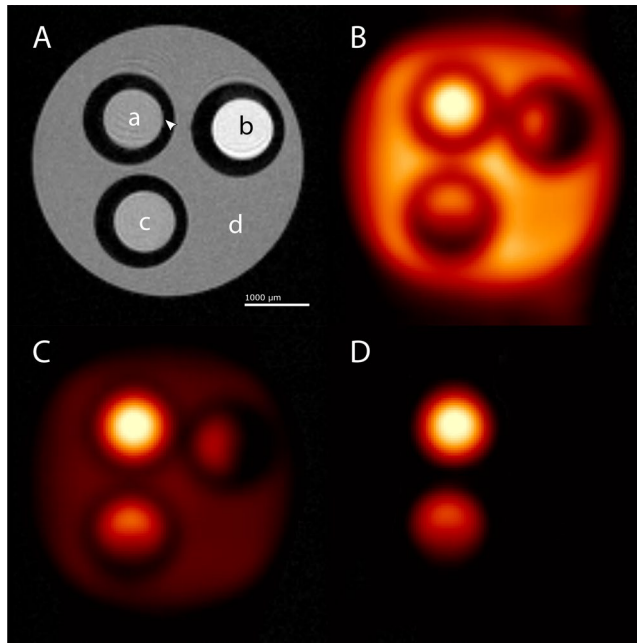


Fig. 5.1 DW-CSI images of reference samples. (A) Reference MSME Image showing three capillaries filled with olive oil (a), hexadecane (b) and oleic acid (c), surrounded by distilled water (d). Note the chemical shift artefact, caused by the different resonant frequencies of fat/oil relative to water, which displaces the hydrocarbon signal to the right, indicated with a white arrowhead. The artefact is minimised by increasing the receiver bandwidth. (B) Heatmap at minimal gradient strength (54 s mm^{-2}). Some B_0 inhomogeneity is visible in the hexadecane and oleic acid capillaries, possibly caused by susceptibility differences. (C) Heatmap at intermediate gradient strength (740 s mm^{-2}). (D) Heatmap at maximum gradient strength ($11,516 \text{ s mm}^{-2}$). Water is fully attenuated at this gradient strength, as well as hexadecane.

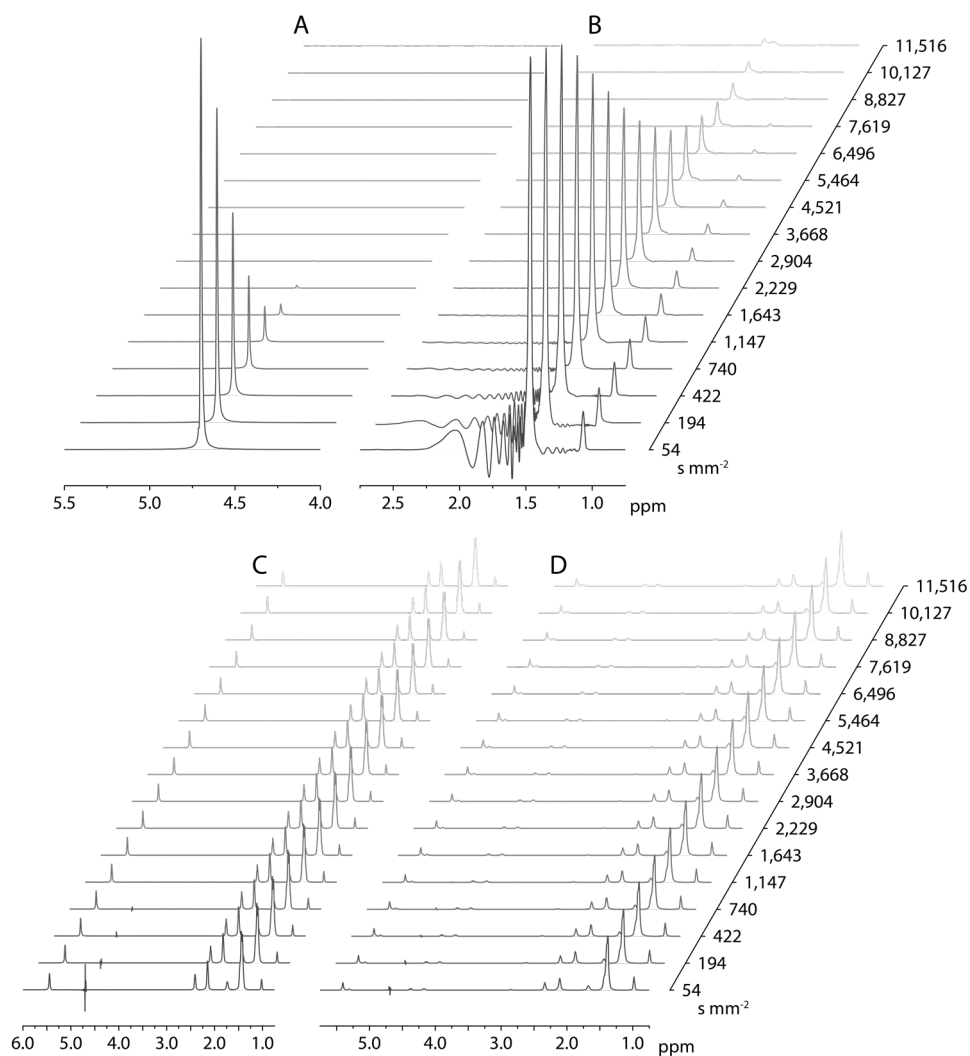
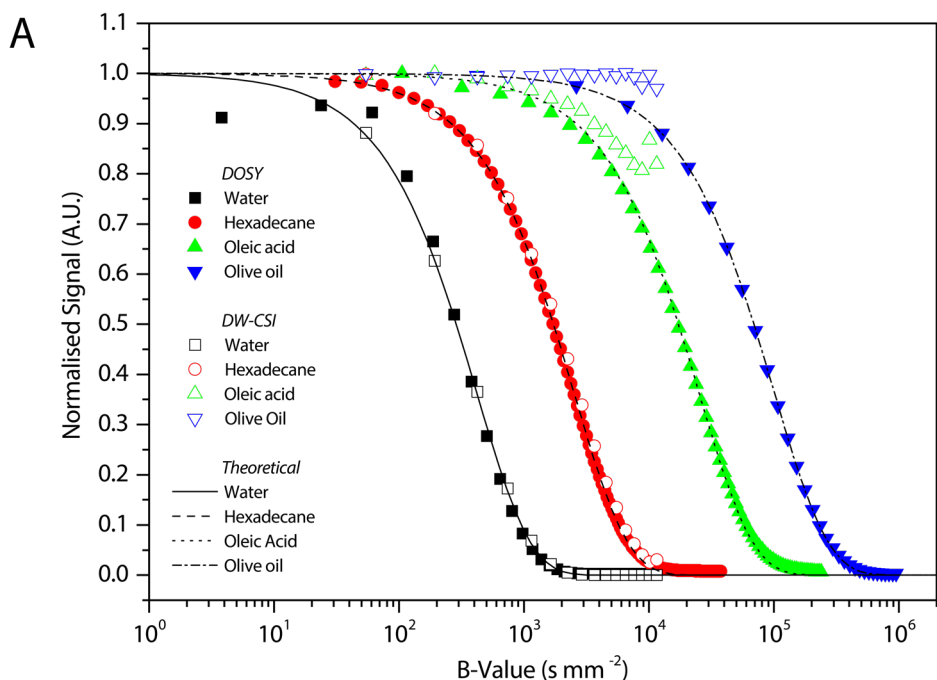


Fig. 5.2 White-washed, stacked plot of DW-CSI reference samples. Each stack depicts a series of representative spectra, with diffusion encoding increasing from front to back. (A) Water spectrum. The resonance at 4.7 ppm is almost completely attenuated at a B-value of 2,904 s mm², reflecting the mobile nature of water at 298 K. (B) Hexadecane gives rise to a primary resonance at 1.5 ppm and a side peak at 1.1 ppm. To the left of each peak, eddy currents or stimulated echo artefacts can be seen. The eddy currents are recognised as a distortion of the baseline with an increasing frequency from left to right. These artefacts disappear as the increasing diffusion gradients cause further spoiling. (C) Oleic acid appears resistant to attenuation at the gradient strength used. Around 4.8 ppm, some artefacts due to 50 Hz noise are visible, which is fully attenuated by increased spoiling at around 1,1147 s mm². (D) Olive oil presents a similar profile to oleic acid due to it being mainly comprised of triglycerides with oleic acid as a base unit. However, unlike oleic acid, its larger molar mass means it diffuses much slower. Olive oil does not appear significantly attenuated at the gradient strength used.



B

Apparent Diffusion Coefficient ($\text{mm}^2 \text{s}^{-1}$)		
Compound	DOSY	DW-CSI
<i>Water</i>	$2.42 \cdot 10^{-3} \pm 5.65 \cdot 10^{-5}$	$2.38 \cdot 10^{-3} \pm 6.81 \cdot 10^{-6}$
<i>Hexadecane</i>	$4.04 \cdot 10^{-4} \pm 1.34 \cdot 10^{-6}$	$3.74 \cdot 10^{-4} \pm 3.18 \cdot 10^{-6}$
<i>Oleic acid</i>	$4.03 \cdot 10^{-4} \pm 2.38 \cdot 10^{-7}$	–
<i>Olive oil</i>	$1.01 \cdot 10^{-5} \pm 2.46 \cdot 10^{-8}$	–

Fig. 5.3: (A) Decay curves of reference samples, plotted as normalised signal versus logarithmic ($\log^{10}$) B-value strength. Because of the broad range in molecular weight (M_w) of the reference sample, diffusion encoding strength required for near full attenuation (0.03 relative intensity) differs widely. Up to 800,000 s mm^{-2} is required for olive oil, while 2,000 s mm^{-2} is sufficient for water. DOSY measurements are indicated with closed symbols; DW-CSI results use open symbols. An ideal mono-exponential decay curve is drawn for each sample, based on the ADC calculated for DOSY results. For water and hexadecane, The DW-CSI decay curves correspond well to those of the DOSY measurements. However, for oleic acid and olive oil, the decay curves deviate significantly from the ideal decay curve. As such, no meaningful ADC could be calculated from oleic acid and olive oil DW-CSI data. (B) Resulting ADC values calculated from DW-CSI and NMR DOSY measurements are listed.

The ADC values may then be calculated by mono-exponential fitting of the signal integrals from a series of spectra with increasing B-values (Fig. 5.3A)., Additional NMR DOSY measurements were performed in a separate experiment, to compare the decay

curves from DW-CSI. Ideal decay curves are drawn for the ADC values as determined by DOSY. The calculated ADC values are listed in Fig. 5.3B.

The ADC values for water and hexadecane were found to differ 1.7% and 8.0% respectively, from those measured by DOSY. The standard deviation reflects the closeness of the fit and does not include systematic errors such as possible temperature differences between measurements (Le Bihan *et al.*, 2006). For the higher weight compounds, oleic acid and olive oil, there is an apparent discrepancy between the DOSY and DW-CSI data. Oleic acid is only attenuated by 17.8% compared to the initial signal intensity at a high B-value of $11,516 \text{ s mm}^{-2}$, leading to a poor fit that does not provide a meaningful ADC value. Likewise, olive oil is not significantly attenuated at the maximum B-value, while DOSY results show clear attenuation. Several factors may contribute to the observed anomalous results, including sample susceptibility differences and eddy currents induced by the gradients.

The current implementation of the DW-CSI sequence does not include eddy current compensation, without which eddy currents can distort or drown out the signal in non-ideal circumstances. An example of this can be seen as line-shape twisting in hexadecane (Fig. 5.2B). Eddy currents can also induce geometric distortions in (DW) images as well over- or underestimation of B-values (Le Bihan *et al.*, 2006). Another cause for the line shape artefact may be a stimulated echo which is caused by unwanted refocussing of residual magnetisation remaining from a previous excitation. Improved phase cycling could reduce the occurrence of such stimulated echoes. Fortuitously, the diffusion encoding gradient in DW-CSI also acts as an additional source of spoiling, reducing stimulated echoes as B-values increase. The extra spoiling causes artefacts to disappear as diffusion encoding increases (Fig. 5.2B).

Olive oil consists of a collection of large compounds (for example, oleic acid triglyceride, $885.43 \text{ g mol}^{-1}$). Its size causes olive oil to require B-values of up to $800,000 \text{ s mm}^{-2}$, in order to be sufficiently attenuated (Fig. 5.3). These high B-value requirements, resulting in long encoding times, could become limiting in samples with high sample susceptibility inhomogeneity. On *in-vivo* samples, this could cause the signal to be dephased before sufficient encoding has taken place. The issue is confounded by the additional time taken up by the diffusion module in a comparatively long CSI sequence. In effect, this currently limits DW-CSI to water or small-molecule diffusion for *in-vivo* measurements on the spectrometer used in this study.

The feasibility of calculating ADC's from DW-CSI measurements has been shown for water and hexadecane. In a separate experiment, 256 mM hexadecane could be detected in 18 m 42 s. Concluding, the DW-CSI sequence is an elegant way of combining information derived from diffusion weighting of secondary metabolites in a spatially resolved manner. However, further research and development, both in sequence and hardware design, is required to utilise this approach to its full potential.

5.3 APPLICATIONS OF SOLENOID MICROCOILS TO PLANTS

In chapter 3, a step-by-step description was provided for testing and characterising solenoid microcoils. The microcoils used in this thesis have a sensitivity up to 4 times that of a 5 mm birdcage resonator (Krug *et al.* 2019, submitted). Given the simplicity and relatively low cost of solenoid microcoils, with excellent performance characteristics, one might expect a wider acceptance of solenoid microcoils by the MRI community than currently seen. In part, this may be facilitated by access to protocols for designing, testing and using microcoils, which this chapter has sought to ameliorate by describing the required knowledge and equipment on a practical level using video. A video publication is especially suited for this purpose due to its inclusive nature, where details are included that authors typically may not consider worthy of description in a written report, and which are nonetheless crucial to successful reproduction of the technology.

Comparing coil performance across different systems is often challenging due to differences in magnetic field strengths, gradient system, consoles, pulse sequences, and choice of parameters, among others. Therefore, the protocol includes a volume-based SNR measure based upon a standardised MSME sequence and sample. In this manner, coils across widely differing systems may be compared, which is facilitated by an initial list of coil SNRs measured across different field strengths (Krug *et al.* 2019, submitted).

The coil demonstrated in the video incorporates a reservoir containing Fomblin as a susceptibility matching fluid. Susceptibility matching is a common strategy for improving microcoil B_1 homogeneity (Olson *et al.*, 1995; Behnia and Webb, 1998; Grant *et al.*, 2001). However, sample induced susceptibility differences, as opposed to those introduced by the coil, become increasingly critical at ultra-high field strengths. The inclusion of microscopic air pockets in roots and microscopic bubbles in capillaries causes fast T_2^* relaxation, with adverse effects on image quality. Vacuum treatment is necessary to achieve distortion-free images at high resolutions. The vacuum treatment yields significant improvement in image quality and SNR compared to non-treated samples, highlighting the importance of sample preparation for MRM.

5.4 CELLULAR RESOLUTION ON *MEDICAGO TRUNCATULA*

In Chapter 4, root nodules of *M. truncatula* were studied using a home-built solenoid resonator (ID 1.5 mm). Although the high resolution $7 \times 7 \times 7 \mu\text{m}^3$ combined with a relatively large field of view $2.4 \times 1.4 \times 1.4 \text{ mm}^3$ is useful, the addition of localised spectroscopy makes MRM unique as an investigative tool.

Practical limitations include the long acquisition times for cellular resolutions requiring up to 28 hours, which implies a trade-off between low throughput, high resolutions on single colonies, or lower resolution on multiple samples. Thus, further improvements in sensitivity are desirable, to allow more colonies to be measured in a reasonable time.

The assignments of resonances, especially within the congested carbohydrate region, was confirmed with 2D extraction NMR. In principle, this could also be achieved *in-situ*

with a Localised COSY (LCOSY) or J-resolved sequence (JPRESS). For 2D sequences, the necessary increments in the indirect dimension cause an increase in measurement time, compared to 1D sequences (Ryner, Sorenson and Thomas, 1995; Braakman *et al.*, 2008). Lastly, DW-MRS or DW-CSI could provide information on the localisation of metabolites on a cellular level, *e.g.*, whether metabolites are present within cells or the intracellular space, and overcome the problem of spectral congestion (Kruiskamp *et al.*, 2002; Wang *et al.*, 2017).

The next step for investigating root tissue would be to create a whole-plant life support system, allowing for longitudinal imaging. Several challenges remain, mainly related to the conditions of the root section within the resonator. Currently, liquid PFD is used to match sample susceptibility and remove air bubbles from the sample. For *in-vivo* studies, it would be desirable to avoid submersion of the root, in order to emulate natural root conditions more accurately. Furthermore, PFD evaporates rapidly and is a potent greenhouse gas (Tsai, 2011). Thus, alternative sample treatment and the introduction of life support systems would be needed to perform *in-vivo* imaging. These changes represent a non-trivial challenge to overcome for *in-vivo*, longitudinal root imaging.

5.5 OUTLOOK

The resolution achievable by MRM continues to improve. However, physical limitations come into play at ultra-high resolution. At imaging resolutions in the range of 10 μm , diffusion attenuation of the signal becomes a limiting factor (Keevil *et al.*, 1998). Diffusion attenuation is illustrated by the resolution dependence of T_2 values recorded by T_2 mapping at 22.3 T (Krug *et al.* 2019, submitted). This limitation is confounded by the exceedingly short T_2^* relaxation rates, in the order of ms, observed in biological samples at high or ultra-high field strengths, *e.g.* 17.6 or 22.3 T. This introduces the need for pulse sequences designed specifically for the challenges of ultra-high field, such as susceptibility differences and the need for accurate measurement of tissue T_2 relaxation. Additionally, high-resolution imaging and DW imaging require strong gradient encoding. Encoding of these gradients needs to be accomplished before the signal has fully relaxed due to T_2 relaxation. Since the sample T_2 limits the gradient durations, the only recourse is to increase the gradient strength. Currently, the commercially available gradients used here reach a maximum gradient strength of 3 T m^{-1} with 60 A gradient amplifiers. Therefore, the most significant performance enhancements possible for MRI at ultra-high field lies in improved gradients, both in terms of resolution and diffusion encoding.

Next, *in-vivo* studies are likely to become more viable in future research. Continuous flow systems have been demonstrated (Kc *et al.*, 2010), which opens the possibility of growing single colonies in longitudinal studies. Moreover, by varying medium flow rate and composition, different stressing conditions could be investigated. Examples include saline, pH and (micro)nutrient deficiency stresses. The addition of a light source, for example, directed through flexible fibreglass, could also provide the options for controlling irradiation regimes.

Microcoils will continue to be miniaturized with increasingly integrated designs. A promising approach is lab-on-a-chip style flow cells, which incorporate both RF resonator and sample holding cell or flow chamber. A flow chamber built around a cryogenic resonator has also been reported (Koo *et al.*, 2011). Often constructed out of transparent materials, these coils are capable of co-registration with for example optical microscopy. The integration of various approaches to MRM in new coil and gradient designs seems to be the most promising avenue for MRM development.

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APPENDIX

DW-CSI MATERIALS & METHODS

A phantom was constructed to evaluate sequence performance, consisting of a 5 mm NMR tube filled with demineralised water and three flame-sealed capillaries. The three capillaries contained hexadecane, oleic acid (Sigma-Aldrich, St. Louis, Missouri, USA) and a generic extra virgin olive oil, respectively. Hexadecane was chosen due to its property of being the longest chain alkane to have a freezing point below room temperature (293K).

DIFFUSION WEIGHTED CHEMICAL SHIFT IMAGING

CSI employs two orthogonal phase encoding steps with pulsed gradients to record a pure spectroscopic echo upon acquisition, instead of a conventional readout gradient used in imaging. DW-CSI adds to this by introducing a pair of diffusion sensitising gradients interspersed around the first excitation pulse and second refocusing pulse. Thus the gradient separation is linked to the interpulse timing of the CSI itself.

For the reference sample experiments, a diffusion gradient duration (δ) of 4 ms used in combination with a diffusion gradient separation of 6.92 ms. Gradient strength was increased from 5% to 80% of maximum gradient strength in 16 steps, achieving a maximum gradient strength of 11,516 s mm⁻². A Hanning function weighted k -space acquisition scheme was utilised, as implemented by the Bruker '*weighted*' measuring method, for an improved Spatial Response Function (SRF). Basic parameters were as follows: TR = 1150 ms; TE = 15 ms; Matrix = 16 × 16; FOV = 5 × 5 mm²; slice thickness = 1 mm; resolution = 312 × 312 × 1000 μm³; number of scans = 2,048; dummy scans = 4. Acquisition time was 39 m 19 s for each diffusion strength increment. Data were reconstructed into a 32 × 32 matrix with linear smoothing for display. Excitation and refocusing were achieved using Sinc3 pulses with a bandwidth of 8000 Hz, centred on the water resonance. Echoes were captured into 4096 points in 511 ms and spectral width was 8012 Hz (10.68 ppm). Magnetic field homogeneity in the selected volume was optimised by shimming the water resonance. Metabolite heatmaps were generated using a quadratic smoothing function.

DOSY NMR

For each compound, a separate sample was prepared and measured on a 600 MHz Avance III NMR spectrometer equipped with a TXI broadband probe. A stimulated echo sequence incorporating bi-polar gradients was used with the following basic parameters: spectral width was set 9.0 ppm or 5411 Hz; 16384 points were acquired with a dwell time of 92.4 μs in digital quadrature mode (DQD); total acquisition time was 1.51 s; number of averages was 8, as well as 8 dummy scans. The diffusion gradient duration was set individually for each compound to ensure sufficient attenuation at 95% diffusion sensitising gradient strength. The following parameters were used for each compound.

Distilled Water: 32 gradient strength increments, $\Delta = 0.2$ s, $\delta = 2.4$ ms, TR = 2 s; Hexadecane: 128 increments, $\Delta = 0.2$ s, $\delta = 5.0$ ms, TR = 10 s; Oleic acid: 64 increments, $\Delta = 0.5$ s, $\delta = 8.0$ ms, TR = 2 s; Olive Oil: 32 increments $\Delta = 0.5$ s, $\delta = 16.0$ ms, TR = 2 s.

DATA PROCESSING

For each reference compound, a series of spectra were extracted from the DW-CSI results using the 'CsiExtract1dSer' macro present in Paravision 5.1 (Bruker Biospin GmbH, Ettlingen, Germany). Stacking of spectra from DW-CSI and DOSY took place in MestReNova (MestreLab Research S.L., Santiago de Compostela, Spain) where peak integrals were calculated for each gradient strength increment. Spectra were zero-filled to 16,384 points and manually phased. Line broadening was 5 Hz. Consequently, tabulated data was exported to Origin Pro for fitting and normalised to facilitate comparison. Because the reference samples used are pure compounds, containing only one faction, *i.e.*, water (w) or hydrocarbons (h), only mono-exponential fitting is required to accurately describe the system: $S(b) = f_h e^{-bD_h}$, where b is the B-value used and D_h the apparent diffusion coefficient (ADC). The mono exponential fitting was performed in OriginPro 9.1.0 with Levenberg-Marquardt algorithm iteration (OriginLab Corporation, Northampton, Massachusetts, USA).

SUMMARY

This thesis investigates the relations between metabolism and anatomy through the use of Magnetic Resonance Imaging (MRI). Two model systems are studied: *Botryococcus braunii*, a green oleaginous algae and *Medicago truncatula*, a small leguminous plant in symbiosis with *Sinorhizobium meliloti* bacteria. Understanding the processes within the studied model systems may potentially aid in answering challenges facing humanity and its environment, related to energy and food security. In order to map the variation of metabolic profiles across tissues, Magnetic Resonance Imaging (MRI) is used extensively, due to its ability to non-invasively study both anatomy and spectroscopy. Dedicated pulse sequences and home-built microcoils are developed for studying these organisms.

Chapter 1 provides a general introduction to the field of metabolic imaging using MRI, as well as the general principles and theoretical background of MRI. The design and characteristics of solenoid microcoils, used for magnetic resonance microscopy, are discussed. The range of pulse sequences used for magnetic resonance microscopy, localised spectroscopy, and combinations thereof, are covered briefly.

Botryococcus braunii is an oleaginous green alga with the distinctive property of accumulating high quantities of hydrocarbons per dry weight in its colonies. Large variation in colony structure exists, yet its implications and influence of oil distribution and diffusion dynamics are not known and could not be answered due to lack of suitable *in vivo* methods. **Chapter 2** seeks to further the understanding on oil dynamics, by investigating naturally relevant large (700-1500 μm) and extra-large (1500-2500 μm) sized colonies of *Botryococcus braunii* (race B, strain Showa), *in vivo*. A comprehensive approach of chemical shift selective imaging, chemical shift imaging and spin echo diffusion measurements are used to study the algae at high magnetic field (17.6T). Hydrocarbon distribution in large colonies was found to be localised in two concentric oil layers with different thickness and concentration. Extra-large colonies were highly unstructured and oil was spread throughout the colonies, but with large local variations. Interestingly, fluid channels were observed in extra-large colonies. Diffusion-weighted MRI revealed a strong correlation between colony heterogeneity, oil distribution, and diffusion dynamics in different parts of *Botryococcus* colonies.

Dedicated solenoid microcoils are used in this thesis to study small-volume samples at high resolutions. The microcoil design may be adapted and iterated to match the sample of interest optimally. **Chapter 3** describes a method to calibrate such microcoils designed for high-resolution magnetic resonance microscopy (MRM). Calibration of new or home-built microcoils is explained systematically using a reference sample. Steps include a method to determine coil resonant modes and determining the reference pulse power using a nutation curve. Performance characteristics of novel microcoils, including sensitivity and RF homogeneity, are established using standardised pulse sequences. A volume-normalised Signal-to-Noise Ratio is calculated to facilitate coil

performance comparison based on image SNR. Sample preparation and avoiding possible pitfalls are discussed.

Interactions between plants and the microbial & fungal soil flora are crucial for the health of soil ecosystems and food production. Microbe-plant interactions are difficult to investigate *in situ* due to their intertwined relation involving morphology and metabolism. **Chapter 4** describes an approach to overcome this challenge by elucidating anatomy and metabolic profile of *Medicago truncatula* root nodules using Magnetic Resonance Microscopy at 22.3 T. A home-built solenoid MR signal detector with an inner diameter of 1500 μm was used to study individual root nodules. A 3D imaging sequence with an isotropic resolution of $(7\ \mu\text{m})^3$ was able to resolve individual cells and distinguish between cells infected with Rhizobia and uninfected cells. The metabolic profile of cells in different sections of the root nodule was detected using localised spectroscopy and reveals that several metabolites including betaine, asparagine/aspartate and choline, vary across nodule zones. Metabolite distribution was visualised using complementary chemical shift imaging. The technical challenges and outlook towards *in vivo* imaging of nodules and the plant root system are discussed.

Chapter 5 contains the general discussion and outlook for imaging plants and algae using MRM. In addition, initial results for the implementation of Diffusion-Weighted Chemical Shift Imaging (DW-CSI) at high field are discussed. The DW-CSI sequence is an elegant way of combining information derived from diffusion weighting of secondary metabolites in a spatially resolved manner. The feasibility of calculating apparent diffusion coefficients from DW-CSI measurements has been shown for water and hexadecane. However, further research and development, both in sequence and hardware design, is required to utilise this approach to its full potential.

SAMENVATTING

Dit proefschrift beschrijft onderzoek naar de relatie tussen metabolisme en anatomie met behulp van kernspintomografie (MRI). Hiervoor zijn twee modelsystemen gebruikt: *Botryococcus braunii*, een groene olierijke alg en *Medicago truncatula*, een kleine peulvrucht die in symbiose leeft met de bacterie *Sinorhizobium meliloti*. Een beter begrip van de metabole processen in deze modelsystemen kan in potentie een bijdrage leveren bij uitdagingen voor de mensheid op het gebied van voeding en energievoorziening. Om de variatie in metabole profielen tussen verschillende weefsels vast te leggen, is magnetische resonantie microscopie (MRM) gebruikt. MRM is geschikt voor het bestuderen van levende organismen door de niet-invasieve anatomische beeldvorming in combinatie met spectroscopie. Om deze organismen te kunnen bestuderen, werden pulssequenties en zelf gebouwde microspoelen zijn ontwikkeld.

Hoofdstuk 1 bevat een algemene introductie van het veld van de kernspintomografie, de theoretische grondslag en de achtergrond van MRI gebruik. Het ontwerp van solenoïde microspoelen en hun eigenschappen worden beschreven in de context van magnetische resonantie microscopie. De pulssequenties die gebruikt worden voor magnetische resonantie microscopie, alsmede gelokaliseerde spectroscopie en combinaties daarvan, worden bondig besproken.

Botryococcus braunii is een groene alg die rijk is aan olie en de bijzondere eigenschap heeft grote hoeveelheden olie per droog gewicht op te slaan in haar kolonies. Er bestaan grote variaties in koloniestructuur, maar de implicaties hiervan voor de oliedistributie en diffusie-eigenschappen zijn nog onbekend. Door gebrek aan geschikte *in vivo* methoden konden deze vragen tot noch toe niet worden beantwoord.

Hoofdstuk 2 verdiept het inzicht in de olieverdeling van *Botryococcus braunii* (ras B, stam Showa), door het onderzoeken van grote (700-1500 µm) en extra-grote (1500-2500 µm) *in vivo* algen kolonies. Hierbij wordt gebruik gemaakt van een uitgebreide aanpak bestaande uit a) chemische verschuiving selectieve beeldvorming, b) chemische verschuiving beeldvorming en c) spin echo diffusiemetingen op hoge magnetische veldsterkte (17.6 T). Koolwaterstofdistributie in grote kolonies bleek zich in twee bolvormige lagen te bevinden met verschillende diktes en concentraties. Extra-grote kolonies waren erg ongestructureerd en de olie was verspreid door de kolonies met grote lokale variaties. Vloeistofkanalen werden ook geobserveerd in extra-grote kolonies. Diffusie-gewogen MRI toonde een sterke correlatie aan tussen heterogeniteit van de kolonie, oliedistributie and diffusie in de verschillende delen van *Botryococcus* kolonies.

Speciaal ontwikkelde solenoïde microspoelen zijn gebruikt in dit werk om monsters met kleine volumes te bestuderen op hoge resoluties. Iteratieve ontwerp aanpassingen resulteerde in een microspoel die optimaal past bij een monster.

Hoofdstuk 3 beschrijft een methode voor het kalibreren van microspoelen die zijn ontworpen voor hoge resolutie magnetische resonantie microscopie (MRM). Het kalibreren van nieuwe of zelfgebouwde microspoelen wordt systematisch uitgelegd aan de hand van een referentiemonster. Er wordt onder andere een methode beschreven om de resonanties van de spoel vast te stellen en het bepalen van het zogenaamde referentie pulse vermogen met behulp van een nutatiecurve. De prestaties van nieuwe microspoelen, inclusief gevoeligheid en radiofrequentie homogeniteit wordt bepaald met gestandaardiseerde pulssequenties. Een volumegenormaliseerde Signaal-tot-Ruis ratio wordt berekend, om op basis hiervan vergelijkingen tussen spoel eigenschappen mogelijk te maken. De monstervoorbereiding en mogelijke valkuilen worden besproken.

Interacties tussen planten en microbiële & schimmelflora zijn van belang voor een gezond ecosysteem in de bodem en voor voedselproductie. Plant-microbe interacties zijn lastig te onderzoeken *in situ*, door de complexe relatie tussen morfologie en metabolisme.

Hoofdstuk 4 beschrijft een aanpak om deze uitdaging op te lossen door de anatomie en metabole profielen van *Medicago truncatula* wortelnodules te meten met behulp van MRM op een veldsterkte van 22.3 Tesla. Een zelfgebouwde solenoidespoel met een binnendiameter van 1500 μm werd gebruikt om individuele wortel nodules te bestuderen. Een 3D-pulssequentie werd ingezet om een drie-dimensionaal beeld te vormen met een isotrope resolutie van $7 \times 7 \times 7 \mu\text{m}^3$. Het oplossend vermogen was voldoende om individuele cellen te onderscheiden, zowel cellen geïnfecteerd met Rhizobia als niet-geïnfecteerde cellen. Vervolgens is het metabole profiel van cellen in verschillende delen van de wortelnodule gemeten met gelokaliseerde spectroscopie. Een aantal metabolieten, waaronder betaïne, asparagine/aspartaat en choline, variëren in concentraties over de verschillende zones van de wortelknol. De distributie van metabolieten werd gevisualiseerd door middel van chemische verschuiving beeldvorming. De technische uitdagingen en de toekomst van *in vivo* meten in nodules en plantenwortels worden besproken.

Hoofdstuk 5 bevat de algemene discussie over de resultaten van dit proefschrift en het toekomstperspectief van MRM voor toepassing op planten en algen. Daarnaast worden initiële resultaten beschreven van een implementatie van een Diffusie-gewogen chemische verschuiving beeldvorming (DW-CSI) op hoog veld. Deze sequentie biedt een elegante manier om diffusieweging te combineren met gelokaliseerde spectroscopie. De haalbaarheid van het berekenen van de Schijnbare diffusie coëfficiënten (ADC) uit DW-CSI-metingen is aangetoond voor water en hexadecaan. Verder onderzoek en ontwikkeling op het gebied van het ontwerpen van zowel sequenties als instrument-ontwerp is vereist om deze aanpak tot zijn volle potentieel te brengen.

PUBLICATIONS

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CURRICULUM VITAE

My name is Remco van Schadewijk. I was born on the 12th of August 1988 in Lisse, The Netherlands. After finishing gymnasium in 2007, I started my Bachelors in Life Science & Technology at the Delft University of Technology and Leiden University. In 2014 I graduated *cum laude* from the Master Life Science & Technology in Leiden. During my master I directed and produced 'The Dreamgazer', an educative children's film utilising participative writing, for my specialisation in Science Communication & Society.

I started doctoral research on plants and algae using Magnetic Resonance Imaging (MRI) in 2014, at the Solid State NMR group of the Leiden Institute of Chemistry under the supervision of Prof. Dr. A Alia and Prof. Dr. Huub J.M. de Groot. As part of my teaching duties I initiated and produced the educational fiction film 'On Being a Scientist', which was a part of a MOOC on scientific integrity. In 2019 I took up a position as a consultant multimedia at the KU Leuven, Belgium.

My work has been presented at various conferences and symposia: presented orally as well as posters at the BioSolar Cells Annual Meetings (2013, 2014, 2015, 2016) in Ede-Wageningen, The Netherlands. Poster presentation at the 6th Symposium on Ultrahigh Field MR (2015), in Berlin, Germany. Presented posters at BSC meeting and 51st meeting of the NMR-DG (2016) where I was runner-up for the poster prize and presented a poster at the 52nd meeting of the NMR-DG (2017), Geleen. Oral presentation at the Media & Learning Conference (2018, 2019) in Leuven, Belgium. Nomination for MEDEA award 2018.

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