Journal of Visualized Experiments

MRM Microcoil Performance Calibration and Usage Demonstrated on Medicago truncatula Roots at 22 T

--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE61266R2
Full Title:	MRM Microcoil Performance Calibration and Usage Demonstrated on Medicago truncatula Roots at 22 T
Section/Category:	JoVE Biology
Keywords:	MRI; MRM; Magnetic Resonance Microscopy; MRS; microcoils; ultra-high field; localized spectroscopy; root; sample preparation; SNR; solenoid; imaging
Corresponding Author:	Remco van Schadewijk, MSc. Universiteit Leiden Leiden, Zuid-Holland NETHERLANDS
Corresponding Author's Institution:	Universiteit Leiden
Corresponding Author E-Mail:	r.van.schadewijk@chem.leidenuniv.nl
Order of Authors:	Remco van Schadewijk, MSc.
	Julia R. Krug
	Andrew G. Webb
	Henk Van As
	Aldrik Velders
	Huub J.M. de Groot
	A Alia
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$3000)

TITLE:

MRM Microcoil Performance Calibration and Usage Demonstrated on *Medicago truncatula* Roots at 22 T

AUTHORS AND AFFILIATIONS:

Remco van Schadewijk[1], Julia R. Krug[2,3,4], Andrew Webb[5], Henk Van As[2,4], Aldrik H. Velders[3,4], Huub J.M. de Groot[1], A Alia[1,6]

[1] Solid-state NMR, Leiden Institute of Chemistry, Faculty of Science, Leiden University, Leiden, the Netherlands

[2] Laboratory of Biophysics, Wageningen University & Research, Wageningen, the Netherlands

[3] Laboratory of BioNanoTechnology, Wageningen University & Research, Wageningen, the Netherlands

[4] MAGNEtic resonance FacilitY, Wageningen University & Research, Wageningen, The Netherlands

[5] C.J. Gorter Center for High Field MRI, Radiology department, Leiden University Medical Centre, Leiden University, Leiden, The Netherlands

[6] Institute for Medical Physics and Biophysics, Leipzig University, Leipzig, Germany

Corresponding Author:

Remco van Schadewijk

r.van.schadewijk@chem.leidenuniv.nl

Email Addresses of Co-authors:

r.van.schadewijk@chem.leidenuniv.nl

julia.krug@wur.nl

a.webb@lumc.nl

henk.vanas@wur.nl

aldrik.velders@wur.nl

groot_h@lic.leidenuniv.nl

a.alia@chem.leidenuniv.nl

KEYWORDS:

MRI, MRM, Magnetic Resonance Microscopy, MRS, microcoils, ultra-high field, localized spectroscopy, root, sample preparation, SNR, solenoid, Imaging

SUMMARY:

A protocol to study biological tissue at high spatial resolution using ultra-high field magnetic resonance microscopy (MRM) using microcoils is presented. Step-by-step instructions are provided for characterizing the microcoils. Finally, optimization of imaging is demonstrated on plant roots.

ABSTRACT:

This protocol describes a signal-to-noise ratio (SNR) calibration and sample preparation method

for solenoidal microcoils combined with biological samples, designed for high-resolution magnetic resonance imaging (MRI), also referred to as MR microscopy (MRM). It may be used at pre-clinical MRI spectrometers, demonstrated on *Medicago truncatula* root samples. Microcoils increase sensitivity by matching the size of the RF resonator to the size of the sample of interest, thereby enabling higher image resolutions in a given data acquisition time. Due to the relatively simple design, solenoidal microcoils are straightforward and cheap to construct and can be easily adapted to the sample requirements. Systematically, we explain how to calibrate new or home-built microcoils, using a reference solution. The calibration steps include: pulse power determination using a nutation curve; estimation of RF-field homogeneity; and calculating a volume-normalized signal-to-noise ratio (SNR) using standard pulse sequences. Important steps in sample preparation for small biological samples are discussed, as well as possible mitigating factors such as magnetic susceptibility differences. The applications of an optimized solenoid coil are demonstrated by high-resolution (13 x 13 x 13 μm^3, 2.2 pL) 3D imaging of a root sample.

INTRODUCTION:

Magnetic resonance imaging is a versatile tool to noninvasively image a wide variety of biological specimens, ranging from humans to single cells[1–3]. 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[1,3,4]. The study of specimens at resolutions below a hundred micrometers is referred to as magnetic resonance microscopy (MRM)[5]. 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). Several approaches can be pursued to optimize SNR[6]. One approach is to use a higher magnetic field strength, while a complementary approach is to optimize 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 that are ≈0.5-2 mm in diameter (e.g., root tissues), microcoils are useful as the SNR is inversely proportional to the coil diameter[6,7]. Resolutions as high as 7.8 x 7.8 x 15 μm^3 have been attained on animal cells using dedicated microcoils[8]. A variety of microcoil types exist, with planar and solenoid coils most commonly used depending on the application and tissue geometry[9]. Planar coils have high sensitivity close to their surface, which is useful for applications on thin slices. For example, a method designed specifically for imaging perfused tissue has been described for planar microcoils[10]. However, planar coils have a high falloff of sensitivity and no well-defined reference pulse power. Solenoid coils, being cylindrical, have a wider area of application and are more favored for thicker samples. Here, we describe the characteristics of the solenoid coil, a protocol to prepare samples for microcoil MRI, as well as the calibration of a solenoid microcoil (**Figure 1A**).

The solenoid coil consists of a conducting wire coiled, like a corkscrew, around a capillary holding the sample (**Figure 1B**). Microcoil assemblies can be constructed using only enameled copper wire, an assortment of capacitors, and a suitable base for soldering the components (**Figure 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 probe characterization (i.e., the theory of electronics, workbench

measurements, and spectrometer measurements for a variety of coil geometries) have been described extensively elsewhere[7,11–14].

A solenoid coil can be built by keeping in mind design rules for the desired dimensions according to the guidelines described elsewhere[15,16]. In this specific case, a coil was used with an inner diameter of 1.5 mm, made from enameled copper wire, 0.4 mm in diameter, looped around a capillary of 1.5 mm outer diameter. This solenoid is held on a base plate on which a circuit is made, comprised of a tuning capacitor (2.5 pF), a variable matching capacitor (1.5-6 pF) as well as copper connecting wires (**Figure 1A, 1C**). The tuning capacitor is chosen to achieve the desired resonant frequency of 950 MHz, while the matching capacitor is chosen to achieve the maximum signal transmission at an impedance of 50 Ohm. The larger capacitor is variable to allow for finer adjustment. In regular operation, tuning and matching are performed using capacitors in the probe base. The assembled microcoil needs to be mounted on a probe so that it can be inserted into the magnet. An additional holder may be required, depending on the system. Here we use a 22.3 T magnet combination with a Bruker Console Avance III HD in combination with a Micro5 probe. In this case, we used a modified support insert equipped with the necessary connections to connect to the ^{1}H channel of the probe (**Figure 1A**).

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[17]. A reservoir was made from 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 **Figure 2A**.

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 length and power, B_1-homogeneity and SNR per unit volume (SNR/mm^3), are the most practically relevant. SNR/mm^3 is useful to compare the performance of different coils on the same system[18]. 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 characterization of the performance of solenoid microcoils is shown: calibrating the 90° pulse length or power; assessing the RF- field homogeneity; and calculating SNR per unit volume (SNR/mm^3). A standardized spin-echo measurement using a phantom is described to facilitate a comparison of coil designs, which allows for the optimization of distinct applications. Phantom and biological specimen sample preparations, specific for microcoils, are described. The protocol may be implemented on any suitable narrow-bore (≤60 mm) vertical magnet equipped with a commercially available 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 artifacts due to differences in magnetic susceptibility[19]. The effect increases with increasing magnetic field strength[20]. 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. An advantage of this protocol is the potential for subsequent co-registration of MRI data with optical microscopy, 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.

PROTOCOL:

NOTE: This protocol describes procedures for usage and evaluation of coil characteristics of a 1.5 mm inner diameter (ID) solenoid coil (**Figure 1**). 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.

1. Reference sample preparation

1.1. To prepare 100 mL of the sensitivity reference solution, dissolve 156.4 mg of $CuSO_4 \cdot 5\ H_2O$ into 80 mL of D_2O contained in a 100 mL GL45 flask. The copper sulfate reduces both T_1 and T_2 relaxation time, allowing for quicker measurements, while the D_2O prevents radiation damping and saturation effects. Manually stir until solids are completely dissolved.

1.1.1. Adjust volume to 100 mL using deionized water for a final concentration of 1 g/L $CuSO_4$ (anhydrous, 6.3 mM). This concentration is sufficient to shorten T_1 and T_2 relaxation but not too high to be affected by precipitation. Seal the reference sample to prevent changing the ratio of H_2O: D_2O.

1.2. Optionally, connect the probe to a network analyzer, to test if the coil resonates at the desired resonance frequency. Perform an S_{11} reflectance test to measure the frequency range achieved by tuning and for Q- factor measurements as described in detail by Haase et al.[14]. Connect the microcoil to the network analyzer using a co-axial cable. Use a BNC adapter cable if necessary.

1.2.1. Set the center frequency on the network analyzer to the desired resonant frequency,

depending on the intended magnetic field strength for which the coil is designed. Next, set the sweep width to 10 MHz. Adjust the variable capacitor on the microcoil assembly, if present, to the fine-tune the reflectance dip to the desired frequency.

1.2.2. Record the reflectance level at the center frequency and the frequency f_1 and f_2 at the -7 dB level. Use these to calculate the Q-factor at the -7 dB level according to Haase et al.[14]

2. Sample preparation

2.1. If preparing a reference sample for coil calibration, transfer 1 mL of $CuSO_4$ solution to a watch glass dish under a stereomicroscope.

2.2. If preparing a biological sample, transfer 1 mL of perfluorodecalin (PFD) into a watch glass under a stereomicroscope, which will be used to submerge the sample. PFD is used as it can fill air spaces in the specimen, without entering biological cells. It is also not observable by proton MRI. Immediately cover the watch glass with a Petri dish lid to prevent evaporative loss, before the PFD is needed.

NOTE: PFD is highly volatile and a potent long-term greenhouse gas[21]. 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[17].

2.3. Cut capillaries of suitable outer diameter to size, to fit inside the diameter of the microcoil holder (18 mm) and allow for repositioning (**Figure 1C**). Use a ceramic cutter to make an incision every 10-12 mm and break carefully on the incision point.

2.4. If preparing a reference sample, use tweezers and the stereomicroscope to bring a pre-cut capillary in contact with the surface of the $CuSO_4$ solution inside the watch glass, allowing capillary action to fill the capillary.

2.5. If preparing a biological sample, use tweezers and a stereomicroscope, 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.

2.5.1. Carefully extract a five week old whole root system from its growth substrate, such as a perlite soil replacement. Clean the root sample meticulously of rhizosheath. Remove large soil particles using tweezers, and if smaller particles are present, remove them by washing the root system with distilled water. Photograph if needed for future reference. Select and excise a small section of fibrous root free of rhizosheath using a scalpel.

2.5.2. For vacuum treatment, place the sample into a 1.5 mL tube containing a suitable fixative solution. Leave the tube cap off, and then seal the tube with parafilm to seal the opening of the

tube. Then, punch holes in the film with a sharp tool to allow for ventilation of the tube.

2.5.3. Place the sample tube in a vacuum chamber, seal the chamber, and connect a lab membrane vacuum pump to the chamber. Subject the sample to vacuum treatment for up to 30 minutes, to reduce the presence of air pockets within biological samples. Halt the vacuum treatment when no air bubbles are seen escaping the sample.

2.5.4. While looking through a stereomicroscope, use tweezers to submerge the sample in the infiltration medium prepared previously. Wash the sample of potential debris.

2.5.5. Insert the sample into the capillary using tweezers, while both the capillary and the sample are fully submerged to avoid the inclusion of air bubbles. Use a smaller capillary or syringe needle tip as a pushing rod (**Figure 2B**).

2.5.6. Take the sample capillary from the medium watch glass, using tweezers. In the case of PFD, cover the Petri dish lid.

2.6. Shape the tissue paper into a fine point and use it to remove circa 1 mm of liquid from both ends of the capillary.

2.7. Melt a small volume of capillary wax using a wax pen. Apply wax on both sides. The wax will turn opaque when it solidifies. Take care to exclude air bubbles from the capillary (**Figure 2C**).

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

2.8. Afterwards, scrape off excess wax from the exterior of the capillary using a scalpel and wipe clean with fine tissue paper.

3. Mounting the sample

3.1. Place a microcoil underneath the stereomicroscope and insert the sample using tweezers while keeping the microcoil steady (**Figure 2D**).

3.2. Use a rod to center the sample in the microcoil, by sliding the capillary inside the solenoid coil.

3.3. Optionally, apply adhesive tape to fix the position of the capillary.

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.

3.5. Attach the microcoil to the socket of the probe base, while keeping the microcoil upright (**Figure 3A,3B**).

3.6. Carefully slide the triple-axis gradient coils over the microcoil while matching the water-cooling connectors of the gradient to that of the probe base (**Figure 3C**). Turn the screw thread on the probe base to fix the gradient in place.

NOTE: This step applies for a Micro5 probe only. In the case of other systems such as Micro2.5 or Biospect, the gradients are on a separate socket than the coil.

4. Determining coil characteristics

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

4.2. Insert the probe into the magnet and connect the necessary cables: RF transmit/receive cable, water-cooling lines, thermocouple cable and air cooling line.

4.3. Set the desired water-cooling temperature (recommended 298 K) for the water-cooling unit.

4.4. Set the target temperature (298 K) and the target gas flow (300 L/h). 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 testing novel (home-built) coils.

4.5. Connect the probe using a 50 Ω co-axial cable to a network analyzer with a suitably wide sweep width (400 MHz), centered on the intended resonance frequency.

4.6. Observe the resonant modes by adjusting the variable matching and tuning capacitors that are present in the probe base.

4.7. Tune and match the resonant mode to the desired frequency.

4.8. Optionally, determine the coil quality factor (Q-factor) on the network analyzer. One method to obtain the quality factor is to use a coupling network and dividing the center frequency (f_c) by the width of the reflection dip at -7 dB (i.e., $Q = f_c/(f_1 - f_2)$)[14]. Set f_c to the operating frequency of the magnet, while f_1 and f_2 are set to the -7 dB point left and right of f_c, respectively. Some network analyzers have Q-factor determination built-in.

4.9. Initiate a reflectance test on the scanner, usually called 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, start with a high spectral sweep width. In some cases, it might be more convenient to tune and match the coil outside the magnet on a network analyzer.

4.10. Select a shim file for the largest volume coil of the imaging probe if it is available. If starting from a coil that has been used previously, use an available shim file. If both options are not available, start with all shim values set to 0.

4.11. Select the correct coil configuration for the microcoil if it is available in the imaging software (i.e., ParaVision). Otherwise, create a new coil configuration matching the specifications of the coil (e.g., single tuned or double-tuned) according to the manual of the 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 1 W 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.

4.12. Record a nutation curve for a new coil to obtain an indication of the correct RF-power for the coil (**Figure 4**). In case the safe limits for the coil are unknown, start with 10 µs at a low pulse power of 0.6 W and slowly increase the pulse lengths by 1 µs at a time until the signal appears.

4.12.1. Using an FID-experiment in the absence of gradient encoding, vary the RF-pulse length systematically while keeping the pulse power constant. The ideal pulse length is the pulse length, where the signal intensity reaches the maximum. If testing a new coil, use a 10 µs pulse with a very low power first and start increasing the pulse power gradually.

NOTE: 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.

4.12.2. For a coil with a homogeneous B_1-field, like a solenoid coil, determine the 180° pulse where the signal intensity decreases to zero[22].

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.

4.14. Use a localizer scan with 3 slices, one slice in each of the three primary axes, to locate the position of the coil within the magnet. To do this, load a localizer scan from the default library of the spectrometer. Starting with a large field-of-view with no offset is recommended. Perform an automated receiver gain adjustment and manually start the measurement.

NOTE: If the sample is exactly in the center of the gradient system, the localizer scan will show the sample. If the coil or sample is not centered in the image slices or missing, the localizer scan needs to be adjusted, in which case step 4.12 needs to be performed again.

4.15. Alternatively, use a complementary way to find the correct 90° pulse based on image

evaluation. Once an approximate pulse power is found using the nutation curve, adjust the pulse powers gradually to check the image for B_1-field homogeneity. For some coils with an inhomogeneous B_1 field, the 90° pulse power determined using the nutation curve may be overestimated, which leads to overtipping in the desired sweet spot of the coil. In this case, reduce the reference pulse power and check the new images against the previous images (**Figure 5**).

4.16. Manually shim the magnetic field based on the FID signal. A recommended order for initial shimming is Z-Z^2-Z-X-Y-Z-Z^2-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 may result in a stronger correction of the B_0 homogeneity for this coil configuration. Higher-order shims have little effect and may be ignored.

4.17. Calculate a volume-normalized SNR to allow for comparison of microcoil characteristics across different systems, adapted from the manufacturer's protocol[18]. For the microcoils used here, we used a spin-echo sequence with the following parameters: field-of-view (FOV) 6 mm x 6 mm, repetition time (TR) 1000 ms, echo time (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.

4.17.1. Determine the volume normalized SNR (SNR/mm^3) in two steps. First, calculate the voxel volume (V_{voxel})(Eq. 1):

$$V_{Voxel} = D_x \times D_y \times D_{slice} \quad (1)$$

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.

4.17.2. Select the regions of interest to determine the signal intensity (μ_{ROI}) of the sample, and the signal intensity (μ_{noise}) and standard deviation (σ_{noise}) for a region outside the sample (i.e., the noise). The mean signal is taken from the center of the image, while the noise signal is calculated from the corner patches (**Figure 6**). Either the spectrometer control software or general-purpose image processing software may be used for these calculations. Use a single repetition if possible, to maintain comparability between different coils.

4.17.3. Use the values to calculate a volume normalized SNR (Eq. 2):

$$SNR_v = \frac{\mu_{ROI} - \mu_{noise}}{\sigma_{noise} \times V_{voxel}} \quad (2)$$

For the coil used here in combination with the reference solution, using Eq. 2 results in the following solution:

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

NOTE: When comparing the SNR of coils at different magnetic field strengths, the relaxation properties of the phantom need to be measured[23], unless a very long repetition time and very short echo time are used.

4.18. Check for susceptibility problems due to magnetic field inhomogeneities: load and run a multiple gradient-echo (MGE) sequence (**Figure 7**). Magnetic field inhomogeneities due to susceptibility differences are visible in the images with longer 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 visualized (due to air spaces in the sample), as well as B_0 field inhomogeneities introduced by the coil material. Use the following parameters, to be adjusted depending on the specifications of the spectrometer and coil used: TR 200 ms, TE 3.5 ms with 48 echoes spaced 3.5 ms apart, flip angle 30 degrees. Matrix size 128 x 128.

NOTE: If multiple (potential) resonant modes or reflection dips were observed in the resonance (wobble) curve, repeat the above steps for each resonant mode to determine the most sensitive one. Depending on the microcoil, different parts of the microcoil assembly may be prone to unintended resonance modes.

5. High-resolution imaging

5.1. Run a 3D-FLASH experiment with the following parameters: TR 70 ms, TE 2.5 ms, matrix size of 128 x 64 x 64, FOV 1.6 x 0.8 x 0.8 mm, flip angle 30°, and receiver bandwidth 50 kHz.

5.2. Derive the pulse powers from the reference pulse power determined earlier; this is automatic in most imaging software. Determine the receiver gain using automatic adjustments. Adjust the FOV if necessary, covering the whole object in both phase-encoding directions to avoid aliasing. Run a gradient duty cycle simulation, if available on the system, to verify that the duty cycle of the experiment stays within the specifications of the gradient coils.

NOTE: These parameters are specific to the coil used for demonstration; it is important to optimize to the local system specifics.

6. Recovering samples for further study or storage

6.1. Remove the sample capillary from the microcoil.

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

6.3. Use a syringe to wash the sample out of the capillary with a solution of choice. Alternatively, use a glass pusher rod to eject the sample.

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

REPRESENTATIVE RESULTS:

Coil Characterization

Upon successful tuning and matching of a coil, its performance may be characterized by the coil Q-factor, 90° reference pulse, and SNR/mm^3. For the 1.5 mm ID susceptibility-matched solenoid coil demonstrated here, the measured Q-factor(unloaded) was 244, compared to 561 for a 5 mm birdcage coil.

The reference 90° pulse was 12 µs at a power level of 0.6 W; *cf.* 5 µs at 45 W for a 5 mm birdcage coil (**Figure 4** and **Figure 5**). This equates to an RF pulse field strength (B_1), using ($B_1 = \pi/2\gamma\tau$), of 0.53 mT for the microcoil and 1.17 mT for the birdcage coil[14] where y is the gyromagnetic ratio, while tau is the pulse duration. Since the pulse power levels (*P*) differ, coils may be compared in terms of transmit efficiency ($B_1/\sqrt{P}$): 0.69 mT/W$^{1/2}$ and 0.18 mT/W$^{1/2}$ for the microcoil and birdcage respectively[14]. Comparing by a 90° pulse, the microcoil is found to be a factor ≈ 4 times more sensitive than the birdcage coil.

Effect of susceptibility matching

At ultra-high field strengths, sample and coil susceptibility become a dominant factor for image quality, as seen in **Figure 7A,7B**. 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.

High-resolution imaging

A high resolution of 13 x 13 x 13 µm^3 of a *Medicago truncatula* root specimen was attained in 20 hours and 23 minutes (**Figure 8**). 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 complete signal loss.

Symbiotic root nodules of *M. truncatula* may also be imaged using this protocol (**Figure 9**). Using a slightly larger unmatched coil (length circa 3500 µm, inner diameter 1500 µm), images with a resolution of up to 16 x 16 x 16 µm^3 were obtained in 33 minutes.

FIGURE AND TABLE LEGENDS:

Figure 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 and a coil length of 3500 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 fiberglass board (yellow). It is mounted on a commercial holder (grey polymer) that is modified to support the reservoir. (**C**) Solenoid coil design components: 1. solenoid coil, 2. sample capillary, 3. 1.5 pF tuning capacitor, 4. variable matching capacitor, 5. fiberglass base plate, 6. copper wire leads.

Figure 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 µm, 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 ø 0.8 x 40 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.

Figure 3: The component 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.

Figure 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 magnetization 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 µs each time. The maximum signal intensity indicates the 90° pulse, around 12 µs. The 180° pulse may also be determined in this way using the minimum intensity.

Figure 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 µs pulse length. (**B**) 12 µs pulse length, optimal for this coil. (**C**) 13 µs pulse length. (**D**) 20 µs pulse length. If the pulse power is set too high, over-tipping may occur, thereby reducing image intensity in the center 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.

Figure 6: Region of Interest placement. The regions of interest (ROI) for the volume normalized SNR calculation can be seen. The mean sample intensity is taken from an ROI that falls within the reference solution sample. The mean noise and standard deviation are calculated from one or more ROI located in the corners of the image.

Figure 7: 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 the number of echoes 48, echo spacing 3.5 ms, 64 averages, acquisition time 27 m 18 s, flip angle 30°. Field of view was 5 x 5 mm, matrix 128 x 128, resolution 39 x 39 x 200 µm. (**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 artifacts caused by B_0 field inhomogeneity are observed.

Figure 8: 3D imaging of a *Medicago truncatula* root section. (**Top**) FLASH image. Several features of the root section can be distinguished, including the epidermis (e), cortex (c), phloem (ph) 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 x 13 x 13 μm^3. Matrix size was 128 x 64 x 64 and field of view 1.6 x 0.8 x 0.8 mm. Receiver bandwidth 50 kHz. (**Bottom**) 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 x 13 x 13 μm^3. Matrix size was 128 x 64 x 64 and field of view 1.6 x 0.8 x 0.8 mm. Receiver bandwidth 70 kHz.

Figure 9: 3D imaging of a *Medicago truncatula* root nodule. (**Top**) Low-resolution image. Basic parameters were as follows: Repetition time 60 ms, echo time 2.3 ms, 4 averages, acquisition time 4 m. Resolution 31 x 31 x 31 μm^3. Matrix size was 64 x 32 x 32 and field of view 2 x 1 x 1 mm. Receiver bandwidth 50 kHz. (**Bottom**) High-resolution image. Basic parameters were as follows: Repetition time 60 ms, echo time 2.3 ms, 8 averages, acquisition time 33 m. Resolution 16 x 16 x 16 μm^3. Matrix size was 128 x 64 x 64 and field of view 2 x 1 x 1 mm. Receiver bandwidth 50 kHz.

DISCUSSION:

This protocol is best suited to biological samples, as many materials and geological samples have significantly shorter T_2 relaxation times, which cannot be imaged by the sequences used here. Even some biological tissues, which exhibit high sample magnetic susceptibility heterogeneity, can be difficult to image at ultra-high field as the effects are correlated to the field strength[24]. The protocol is not only useful for new coils but may also aid in troubleshooting and diagnosis of potential problems. When testing new or unknown samples, this protocol can be performed beforehand on the reference solution to verify that the experimental setup is functioning according to specifications. This aids in troubleshooting since the spectrometer can be excluded as a source of artifacts and malfunctions. In addition, this sets the tuning and matching capacitors on the probe to values typical for the microcoil.

When no signal is recorded upon the first experiment, the field of view of the localizer scan can be enlarged to check if the sample is seen. Next, recheck if the coil is tuned correctly and attempt another localizer scan. It is possible that the coil exhibits additional unintended resonant modes, in which case the correct one needs to be determined. If still no image can be obtained, remove

the sample to check its position within the microcoil assembly and verify that the sample is intact (i.e., no air bubbles or leaks in the seals are present). Lastly, a sample may be prepared with water instead of PFD. In case the sample gives little detectable signal in the localizer scan, the surrounding water in the capillary can still be detected.

As microcoils are ideally very close to the sample, the magnetic susceptibility differences between the air and the wire can cause additional signal loss, as seen in **Figure 7B**. Potential artifacts include spatial mismapping and anomalous signal intensity variation. Especially gradient-echo type pulse sequences are affected by this non-uniform signal loss. For this reason, we presented a susceptibility-matched coil, by submerging the wire in fluorinert liquid (Fomblin or FC-43). The B_1 estimation method included in this protocol can help determine whether the B_1 susceptibility differences warrant the inclusion of susceptibility matching strategies in the design of the coil assembly. An alternative approach for constructing a susceptibility matched coil is to use susceptibility-matched wire[25]. 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[19] (**Figure 5A**). A critical aspect of 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. It is also important to check that the capillary wax forms a good seal, as dehydration can cause large air bubbles to form.

For the air spaces inside the sample, PFD was used to fill up the intercellular air spaces while not penetrating the cell membranes[26]. 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 noninvasively 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 realize a perfusion setup. First, connecting a medium feed tube as well as a 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[10].

The noninvasive nature of MR imaging, combined with the inert liquid used in this protocol (PFD or Fomblin) means 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 recently demonstrated a combination with optical microscopy on *Medicago truncatula* root nodules[27].

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 good RF homogeneity. Furthermore, spectroscopic imaging can be performed at higher resolutions than otherwise feasible. 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.

ACKNOWLEDGMENTS:

Experiments at the 950 MHz 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 Netherlands' Magnetic Resonance Research School (NMARRS) graduate school [022.005.029]. We thank Defeng Shen and Ton Bisseling for providing the *Medicago truncatula* samples. We further thank Klaartje Houben, Marie Renault, and Johan van der Zwan for technical support at the uNMR-NL facility. We would also like to thank Volker Lehmann, Henny Janssen, and Pieter de Waard for technical help. We express our gratitude to Frank Vergeldt, John Philippi, and Karthick B. Sai Sankar Gupta for their advice. Lastly, we thank Jessica de Ruiter for providing the voice-over to the video.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Ciobanu, L., Pennington, C.H. 3D micron-scale MRI of single biological cells. *Solid State Nuclear Magnetic Resonance*. **25** (1–3), 138–141 (2004).
2. Aguayo, J.B., Blackband, S.J., Schoeniger, J., Mattingly, M.A., Hintermann, M. Nuclear magnetic resonance imaging of a single cell. *Nature*. **322**, 190–1 (1986).
3. Radecki, G., Nargeot, R., Jelescu, I.O., Le Bihan, D., Ciobanu, L. Functional magnetic resonance microscopy at single-cell resolution in Aplysia californica. *Proceedings of the National Academy of Sciences of the United States of America*. **111** (23), 8667–72 (2014).
4. Lee, C.H. et al. Magnetic Resonance Microscopy (MRM) of Single Mammalian Myofibers and Myonuclei. *Scientific Reports*. **7** (1), 39496 (2017).
5. Callaghan, P.T. *Principles of nuclear magnetic resonance microscopy*. Oxford University Press. Oxford. (1994).
6. Glover, P., Mansfield, S.P. Limits to magnetic resonance microscopy. *Reports on Progress in Physics*. **65** (10), 1489–1511 (2002).
7. Peck, T.L., Magin, R.L., Lauterbur, P.C. Design and analysis of microcoils for NMR microscopy. *Journal of Magnetic Resonance. Series B*. **108**, 114–124 (1995).
8. Lee, C.H., Flint, J.J., Hansen, B., Blackband, S.J. Investigation of the subcellular architecture of L7 neurons of Aplysia californica using magnetic resonance microscopy (MRM) at 7.8 microns. *Scientific Reports*. **5** (June), 11147 (2015).
9. Fratila, R.M., Velders, A.H. Small-Volume Nuclear Magnetic Resonance Spectroscopy. *Annual Review of Analytical Chemistry*. **4** (1), 227–249 (2011).
10. Flint, J.J., Menon, K., Hansen, B., Forder, J., Blackband, S.J. Metabolic Support of Excised,

Living Brain Tissues During Magnetic Resonance Microscopy Acquisition. *Journal of Visualized Experiments*. **2017** (128), 1–10 (2017).
11. Minard, K.R., Wind, R.A. Solenoidal microcoil design. Part I: Optimizing RF homogeneity and coil dimensions. *Concepts in Magnetic Resonance*. **13** (2), 128–142 (2001).
12. Vegh, V., Gläser, P., Maillet, D., Cowin, G.J., Reutens, D.C. High-field magnetic resonance imaging using solenoid radiofrequency coils. *Magnetic Resonance Imaging*. **30** (8), 1177–1185 (2012).
13. Minard, K.R., Wind, R.A. Solenoidal microcoil design Part II: Optimizing winding parameters for maximum signal-to-noise performance. *Concepts in Magnetic Resonance*. **13** (3), 190–210 (2001).
14. Haase, A. et al. NMR probeheads for in vivo applications. *Concepts in Magnetic Resonance*. **12**, 361–388 (2000).
15. Webb, A.G. Radiofrequency microcoils for magnetic resonance imaging and spectroscopy. *Journal of Magnetic Resonance*. **229**, 55–66 (2013).
16. Peck, T.L., Magin, R.L., Lauterbur, P.C. Design and Analysis of Microcoils for NMR Microscopy. *Journal of Magnetic Resonance, Series B*. **108** (2), 114–124 (1995).
17. Olson, D.L., Peck, T.L., Webb, A.G., Magin, R.L., Sweedler, J. V *High-Resolution Microcoil 1H-NMR for Mass-Limited, Nanoliter-Volume Samples*. *Science*. **270** (5244) (1995).
18. Oerther, T. *Micro Imaging Manual for AV3 Systems*. Bruker Biospin GmbH. Rheinstetten, Germany (2012).
19. Callaghan, P.T. Susceptibility and Diffusion Effects in NMR Microscopy. *Encyclopedia of Magnetic Resonance*. (2007).
20. Donker, H.C.W., Van As, H., Snijder, H.J., Edzes, H.T. Quantitative 1H-NMR imaging of water in white button mushrooms (Agaricus bisporus). *Magnetic Resonance Imaging*. **15** (1), 113–121 (1997).
21. Tsai, W.T. Environmental property modelling of perfluorodecalin and its implications for environmental fate and hazards. *Aerosol and Air Quality Research*. **11** (7), 903–907 (2011).
22. Keifer, P.A. 90° pulse width calibrations: How to read a pulse width array. *Concepts in Magnetic Resonance*. **11** (3), 165–180 (1999).
23. Vlaardingerbroek, M.T., den Boer, J.A. *Magnetic Resonance Imaging: Theory and Practice*. doi: 10.1007/978-3-662-05252-5. Springer-Verlag. Berlin, Heidelberg. (2003).
24. Schenck, J.F. The role of magnetic susceptibility in magnetic resonance imaging: MRI magnetic compatibility of the first and second kinds. *Medical Physics*. **23** (6), 815–850 (1996).
25. Kc, R., Henry, I.D., Park, G.H.J., Aghdasi, A., Raftery, D. New solenoidal microcoil NMR probe using zero-susceptibility wire. *Concepts in Magnetic Resonance Part B: Magnetic Resonance Engineering*. **37B** (1), 13–19 (2010).
26. Littlejohn, G.R., Gouveia, J.D., Edner, C., Smirnoff, N., Love, J. Perfluorodecalin enhances in vivo confocal microscopy resolution of Arabidopsis thaliana mesophyll. *New Phytologist*. **186** (4), 1018–1025 (2010).
27. van Schadewijk, R. et al. Magnetic Resonance Microscopy at Cellular Resolution and Localised Spectroscopy of Medicago truncatula at 22.3 Tesla. *Scientific Reports*. **10** (1), 971 (2020).

Figure 1

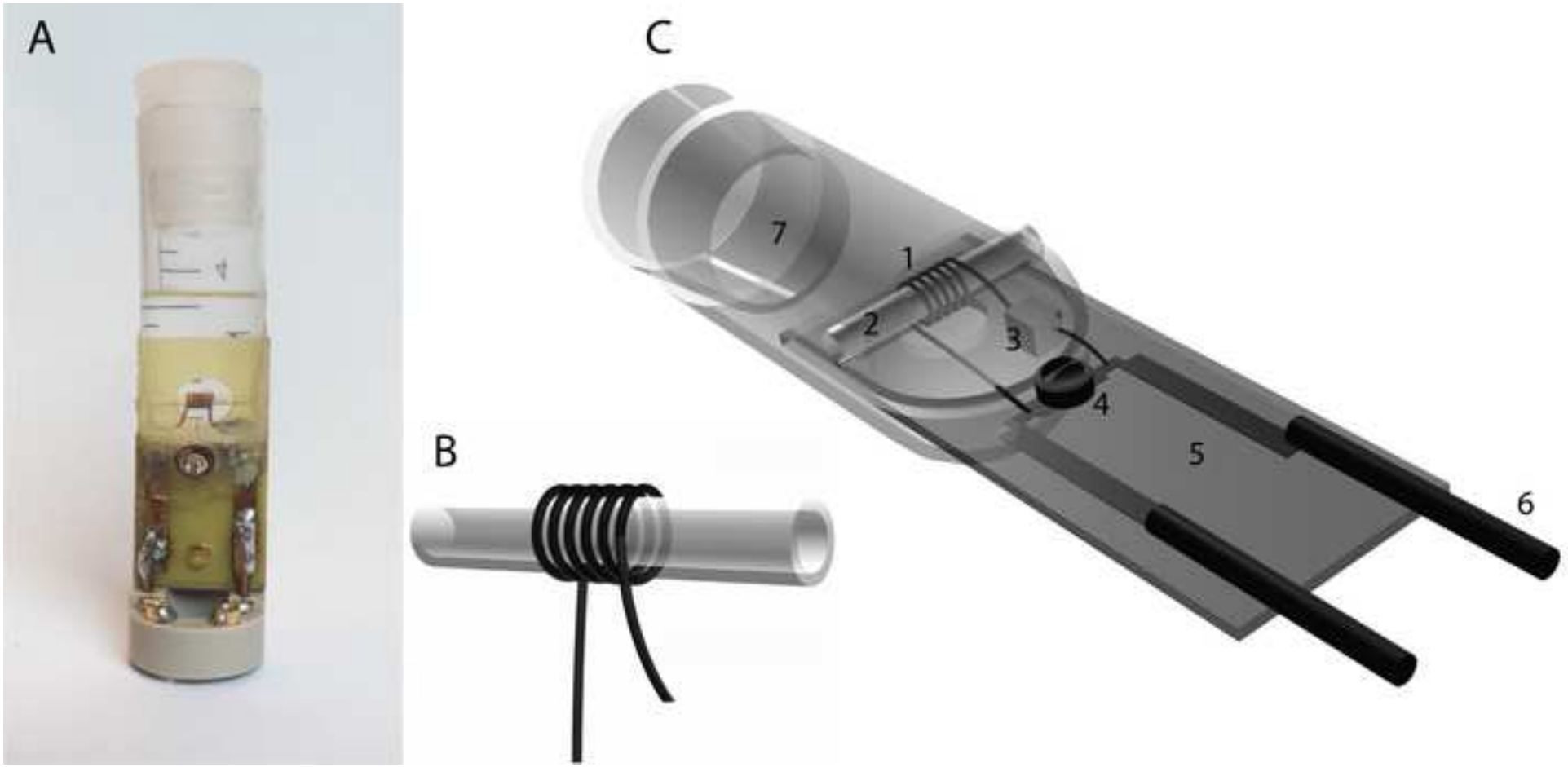


Figure 2

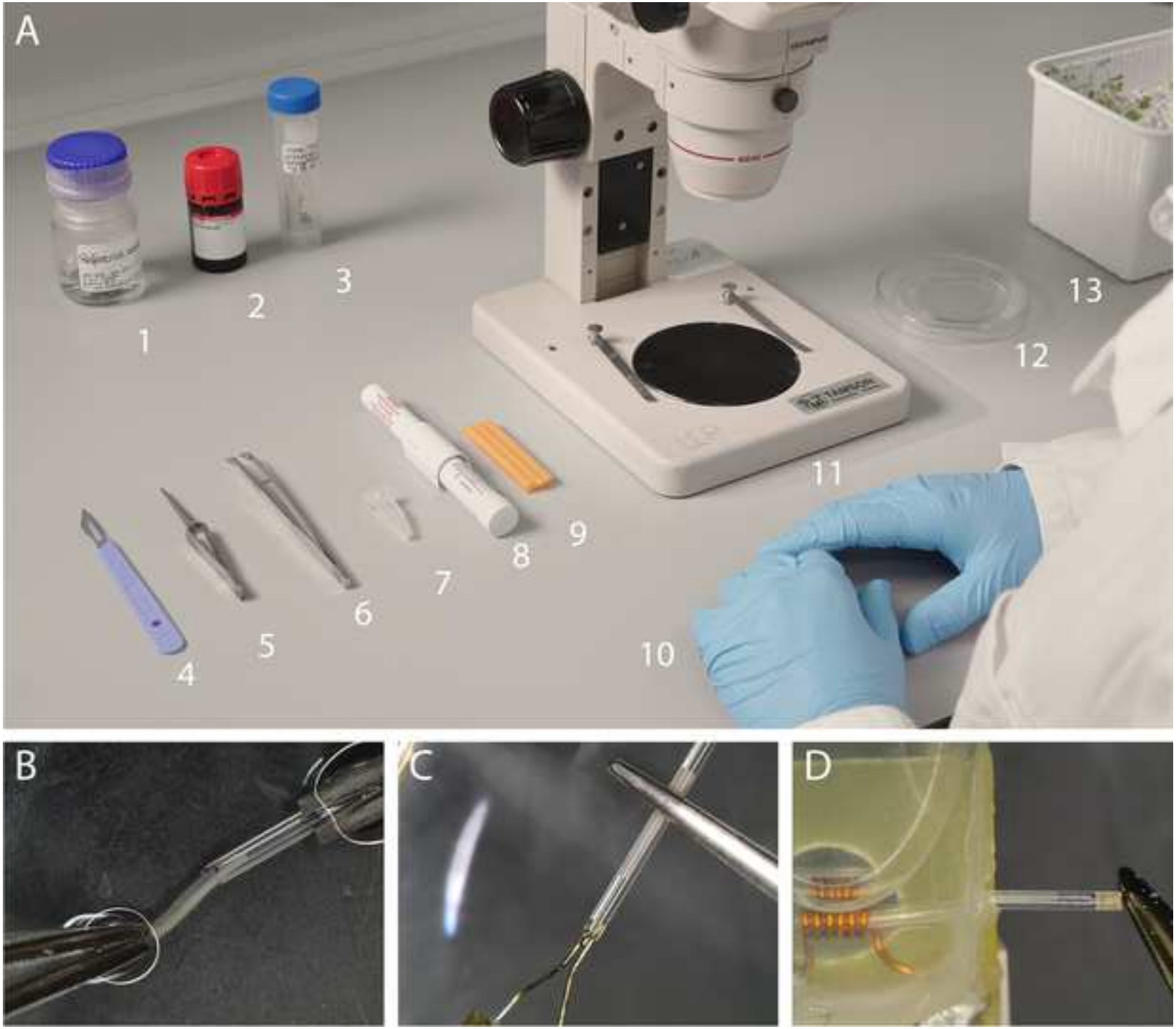


Figure 3

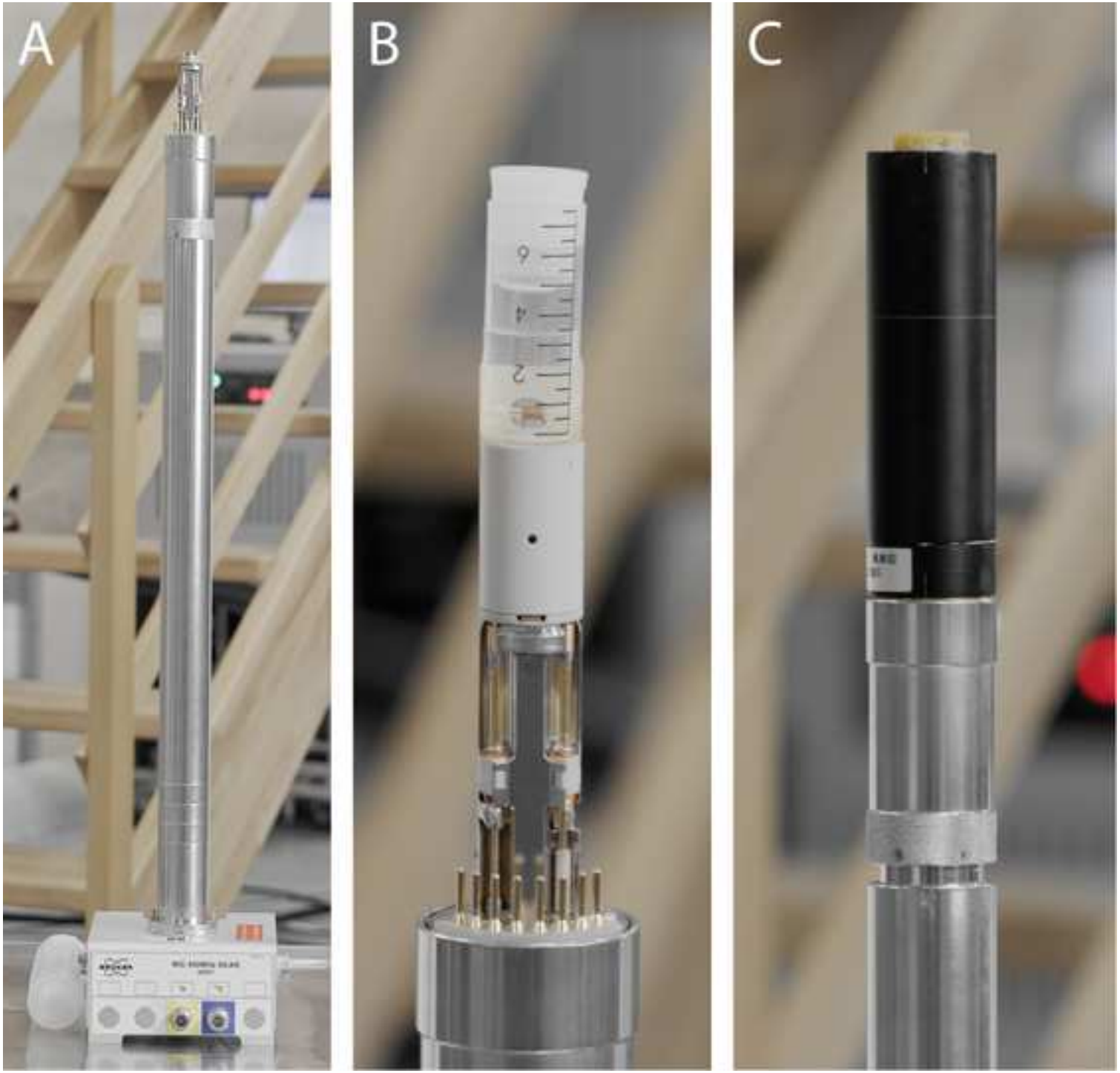


Figure 4

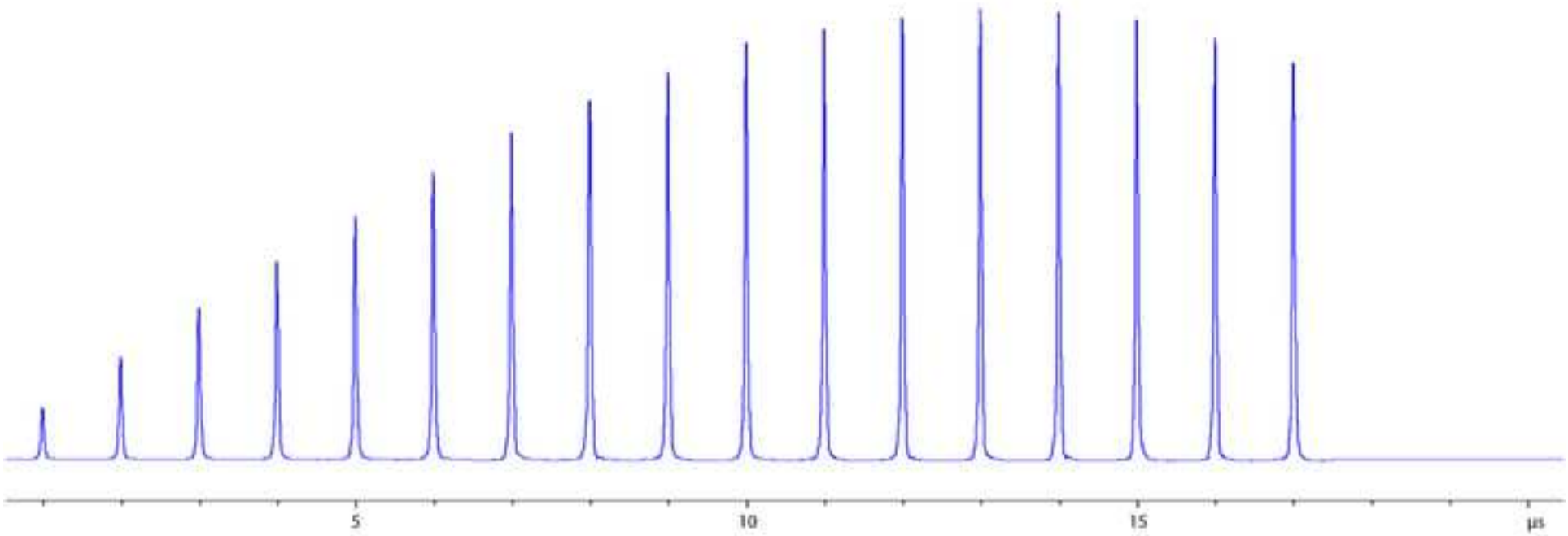


Figure 5

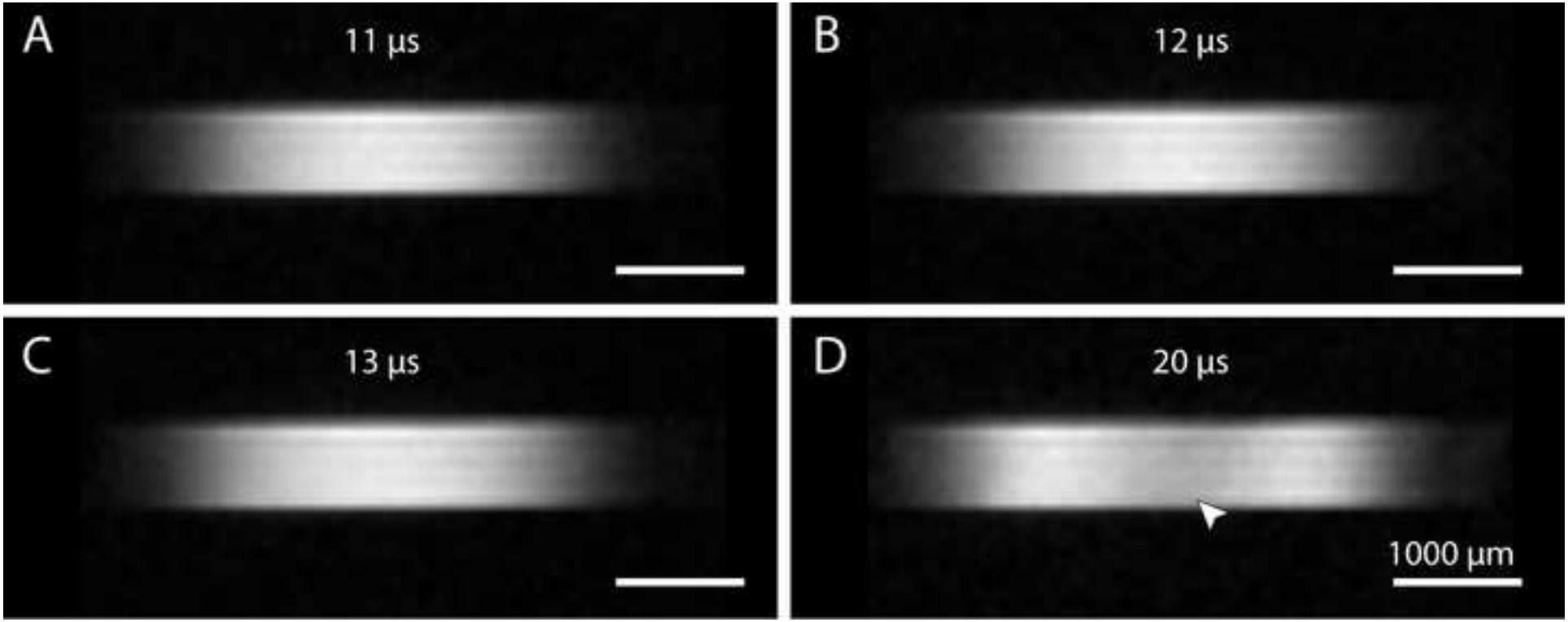


Figure 6

Click here to access/download;Figure;Manuscript_Figure 6.tif

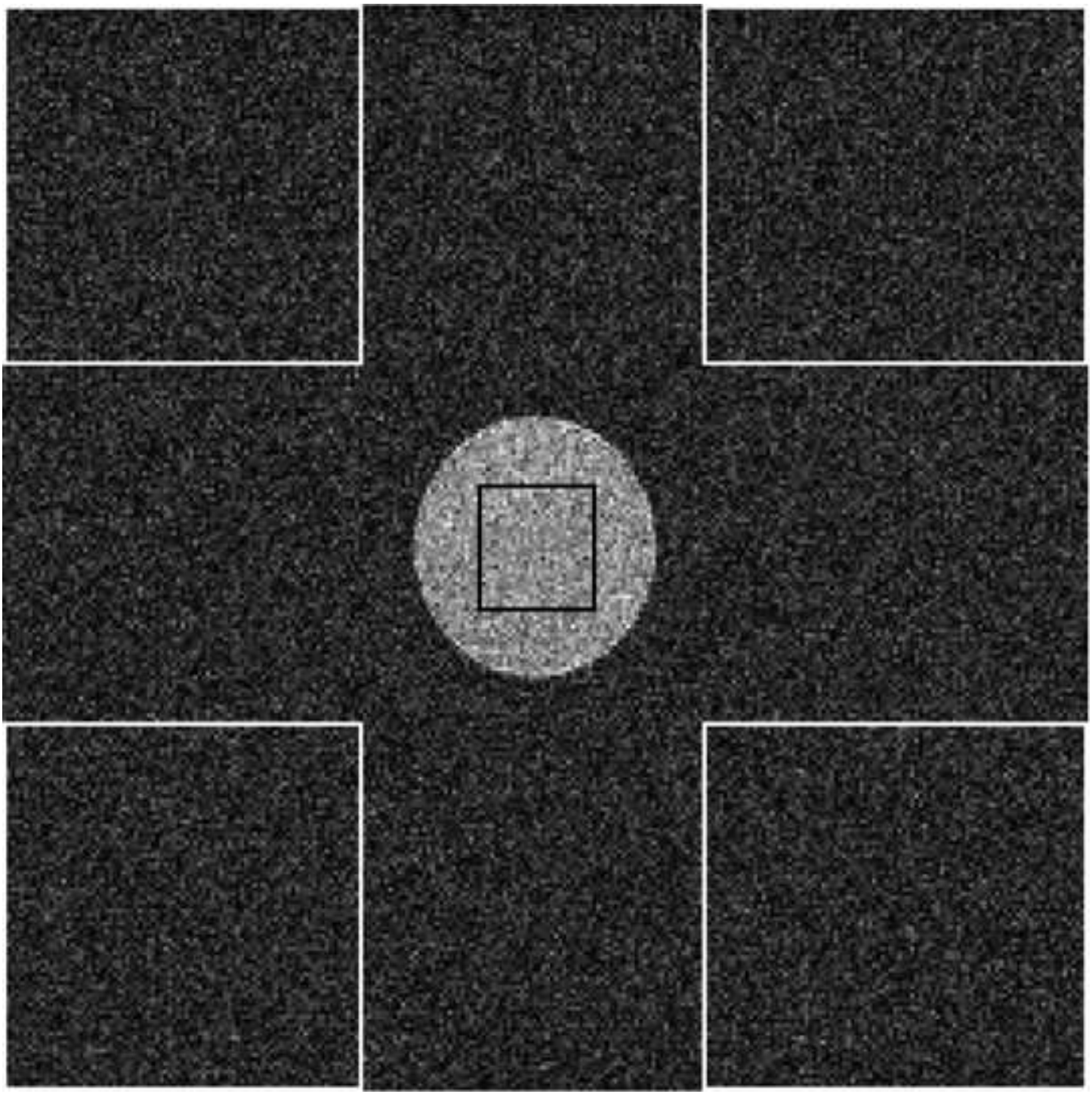


Figure 7

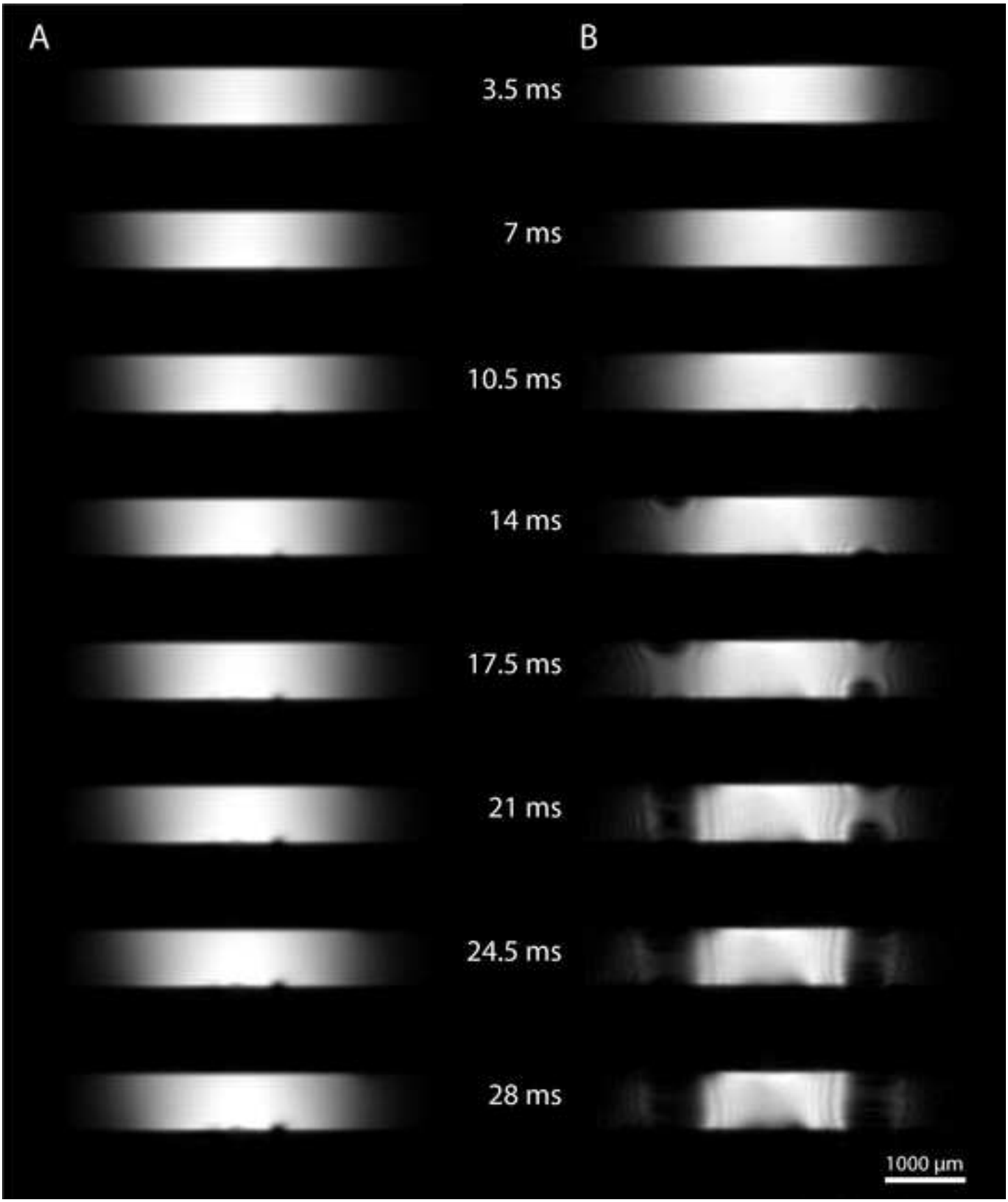


Figure 8

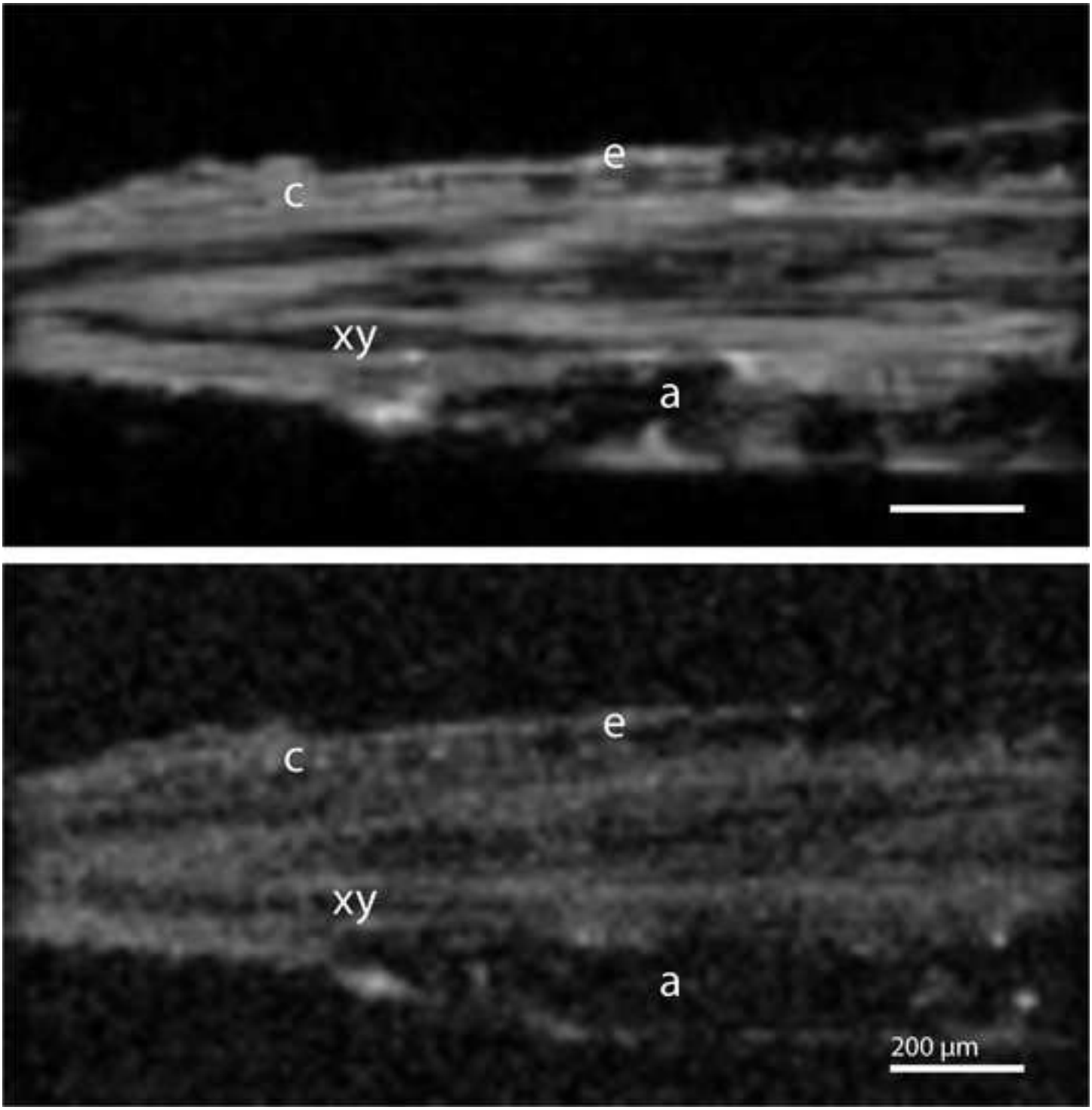
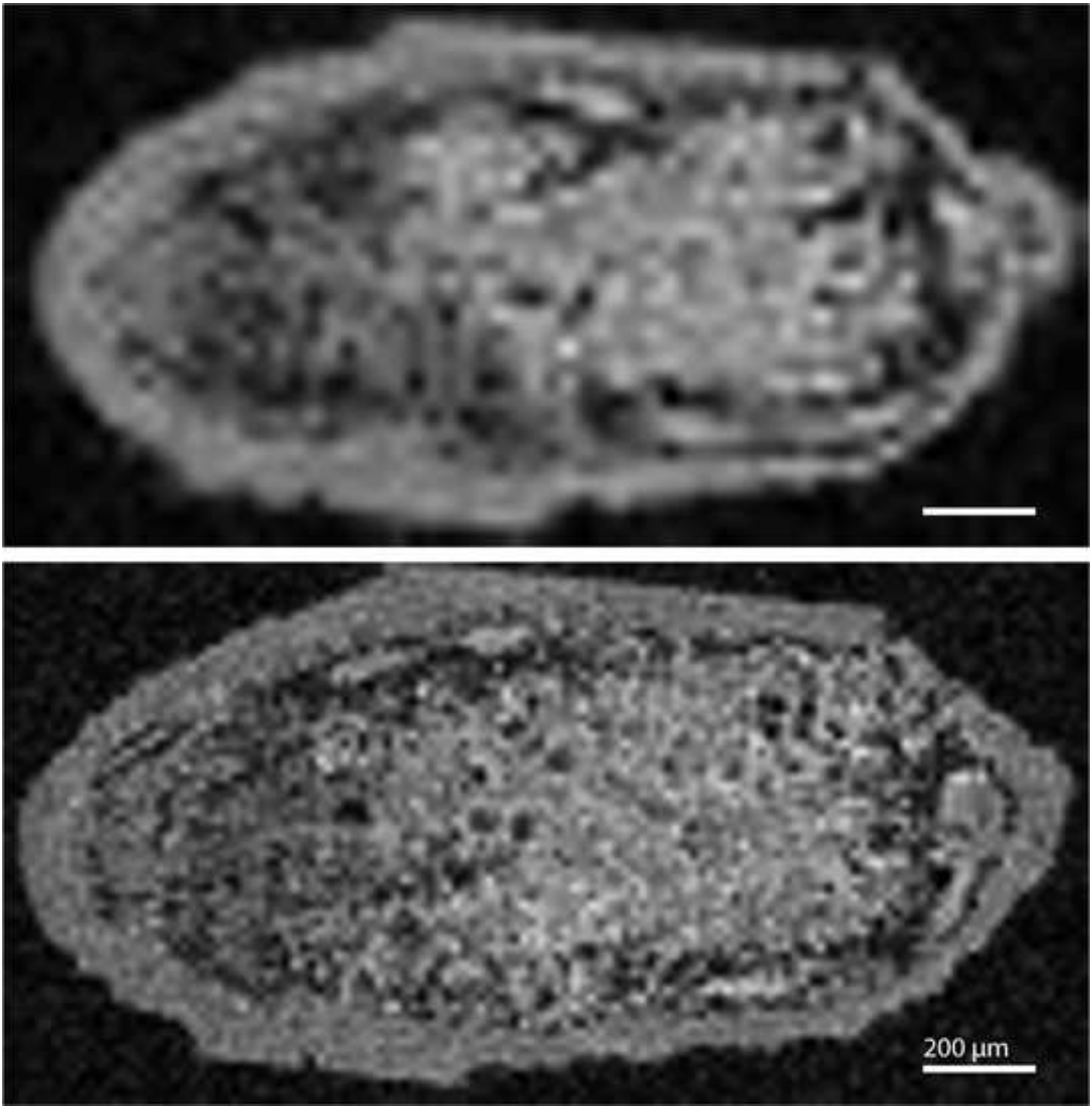


Figure 9



Name of Material/ Equipment	Company	Catalog Number
Reference solution preparation		
$CuSO_4$	Sigma-aldrich	469130
D_2O	Sigma-aldrich	151882
Weigh Scale	Sartorius	PRACTUM513-1S
Sample preparation		
Capillary 1000 µm (Outer diameter)	Hilbenberg GmbH	1408410
Capillary wax	Hampton Research	HR4-328
Disposable Scalpel	Swann-Morton	No. 11
Perfluorodecalin	Sigma-aldrich	P9900
Stereo Microscope	Olympus	SZ40
Syringe	Generic	-
Vacuum Pump	Vacuubrand	MZ2C
Wax pen	Hampton Research	HR4-342
Imaging Hardware		
22.3 T Magnet	Bruker GmbH	950 US^2
Air cooler	Bruker GmbH	-
Console	Bruker GmbH	Avance III HD
Micro5 gradient coils	Bruker GmbH	Mic5
Micro5 Probe body	Bruker GmbH	Mic5
RF microcoil	Home-built	-
Vector Network Analyzer	Copper Mountain Techn	TR1300/1
Water cooler	Bruker GmbH	BCU-20

	Comments/Description
Crystalline powder for creating reference solution	
Liquid used to prepare reference sample	
Scale for weighing compounds	
Sample capillaries	
Solid wax used to seal samples	
Used to excise samples	
Liquid used for submerging sample	
Tabletop binocular microscope	
Used to apply PFD and manipulate the sample	
Two-stage membrane vacuumpump used for removing air pockets from samples.	
Handheld wax pen used to melt and apply capillary wax to samples	
Narrowbore superconducting magnet	
Used to regulate probe temperature	
Controls operation of the spectrometer	
Removable gradient coils mount on the Micro5 probe body	
Holds microcoils and gradient coils	
contains Fomblin	
Used to perform S11 reflectance test, frequency range 300kHz to 1.3 GHz	
Open loop watercooling to dissipate heat from gradient coil operation.	

RESPONSE TO REVIEWERS COMMENTS

Editorial and production comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

****We thank the editor for the useful comments. We have revised grammar and improved grammar where necessary throughout the manuscript.*

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points

****The manuscript has been conformed to the specifications.*

3. Please use American English throughout.

****The spelling has been changed to American English.*

4. Please revise the title to be more concise and avoid punctuation. We cannot have colons in the title - please ensure this change is performed in the video as well.

****As suggested, the title has been made more concise, and the colon has been removed (Line 2-3).*

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

****All text has been rephrased in the imperative tense where applicable.*

6. The Protocol should contain only action items that direct the reader to do something.

****We have changed the protocol accordingly. We have also moved two paragraphs of description of the microcoil from the protocol to the introduction, to indicate more clearly that coil calibration rather than coil construction is the focus of the protocol (Lines 83-108).*

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

****We have carefully gone through the protocol to ensure that large paragraphs have been replaced with individual steps.*

8. Please format the unit as mg/mL and not mg.ml-1.

****All units have been reformatted.*

9. Please ensure you answer the "how" question, i.e., how is the step performed?

****We have gone through the protocol and made it more clear how several actions can be performed, as per the suggestions of the reviewers.*

10. 1.2: How is this done? Please briefly explain the procedure.

**** A brief description of the S_{11} reflectance test has been added to the text with two additional steps (1.2.1 and 1.2.2, lines 178-185) .*

11. Line 196: We cannot have non-numbered sentences in the protocol section.

****We have moved the sentence into step 2.5.3 (Line 226-229).*

12. 4: Please use imperative tense throughout

**** We have changed the tense to imperative where applicable throughout the protocol.*

13. 4.11: Please make subsections and explain how this is done in your experiment.

****The subsections have expanded to increase the clarity of the necessary steps (expanded into 4.11-4.13).*

14. 4.13: How do you obtain the localizer scan?

****We use the default pulse sequence provided by the manufacturer. Some sentences have been added to describe the acquisition of the localizer scan (lines 342-348)*

15. Please ensure that there is a 10-page limit for the Protocol including heading and spacings.

****We have ensured that the protocol is no longer than ten pages (approximately seven pages).*

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

****We have checked the figures and as far as we are aware, all figures in this manuscript are novel and have not been previously published.*

17. As we are a methods journal, please ensure the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

****The most crucial steps in sample preparation have been highlighted, such as the submerged preparation of the sample, exclusion of air bubbles, and check correct wax sealing.*

b) Any modifications and troubleshooting of the technique

****We have included a new paragraph discussing the troubleshooting of measurements, particularly initial signal acquisition (lines 592-599).*

c) Any limitations of the technique

****A brief discussion of the types of usable samples has been added (lines 581-585).*

d) The significance with respect to existing methods

****We have highlighted some of the benefits of microcoil imaging using solenoid coils, such as spectroscopic imaging (lines 643-644).*

e) Any future applications of the technique

****We have added a recommendation to include the techniques as a control in regular experiments to verify that experimental setups are working according to specifications (lines 586-590)*

18. Please do not abbreviate the journal titles in the references section

****All journal titles are now un-abbreviated.*

19. Please sort the materials table in alphabetical order.

*** *The materials have been sorted alphabetically, per section.*

Video:
1. Please ensure that the title is the same both in the video and in the text. Please remove the colon as make the title concise.

****The titles have been harmonised, the colon has been removed and the title is now more concise.*

2. Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text and is in imperative tense throughout as in the text.

****Thank you pointing this out, we have made changes both to the video and manuscript to make more alike and changed it to imperative tense where applicable.*

3. Please ensure that the protocol chapter titles are the same as in the text.

****The protocol and text protocol names have been made the same.*

4. Please remove the numbering from the chapter title cards

****The numbering has been removed.*

5. Please include all the results figure in the representative result section as done in the text. Instead of the application section, please name this section as representative result. Please ensure this section has a voice over.

****The representative results have been moved in the video to the representative results section and the voice over has been amended where necessary.*

6. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

****Some sentences have been added to the voice over of the representative results section to help interpret the findings for the viewer.*

7. Please ensure all the figures are present both in the text and the video.

****We have added a figure to show the ROI placement, so that all figures are present in both forms.*

8. Please include the title card both in the beginning and in the end.

****The title card has been added to the end of the video.*

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript addresses a methodology description for a calibration and a use of microcoils. The methodology includes the bench calibration of the in-house built solenoid coil, SNR calibration in the MR scanner with the sample and extensive sample preparation procedure. The manuscript sufficiently described intro for the protocol with unbiased discussion of the protocol. The efficacy of the protocol is sufficient and could be improved according to the comments below.

Major Concerns:
Some bench calibration and SNR calibration details are not addressed yet.. This info will be helpful for the reproducibility of the all experiment, i.e. targeted S11 and Q-factor values, the choice of the MRI sequence of the SNR calculation and the ROI area for the SNR calculation. In general, the authors followed detailed step-by-step guide for the all procedure. However, the motivation for the choice of some methods are missing. The detailed comments can be found under minor concerns below.

**** We thank the reviewer for the detailed comments and useful suggestions. We have incorporated the suggested changes. Please see our responses under minor concerns.*

Minor Concerns:
Line 44: Maybe it would be beneficial to add one sentence to summarize which environment and which components are included in this methodology description by using who, what, where, how type of questions for general readers in the abstract section. For example, it could include "a SNR calibration for a solenoidal microcoil at the MRI scanner with samples of…"
Line 45: Please be specific about the calibration, such as SNR calibration, coil performance calibration etc.

****We agree with the reviewer that the initial impression on the reader should provide the general details and have introduced this information in the first sentences (lines 45-48).*

Line 75: It could be nice to emphasis a motivation and application area of using planar and solenoid coils depending on the sample type, shape or size.

****We have included some sentences to introduce the main applications for planar and solenoid coils, as suggested by the reviewer (lines 75-79).*

Line 133: Does it mean 0.4 mm-diameter copper wire?

****Indeed, this is the diameter. We have rephrased the sentence to make the diameter explicit.*

Line 135: Is it a variable/trimmer capacitor with the range of values 1.5-6 pF?

****That is correct, we have added 'variable' to the sentence to make this clear.*

Line 133-135: Please add the manufacturer of the copper wire and capacitors on table of materials.

****We thank the reviewer for the comment; the emphasis in this protocol lies on calibration and sample preparation. Though detailed component information may aid in the construction of microcoils, it is likely that this information is too specific and not applicable to the potential users of this protocol.*

Line 145: What is Bruker Micro5 probe? It sounds like very specific to the vendor.

****This is indeed a probebase with an integrated gradient made by Bruker GmbH, we have rephrased the sentence to be vendor-agnostic.*

Line 631: Please check the reference since one author's name was written two times (Ref. 20).

****The reference has been checked and corrected.*

Line 151: Please write it down the motivation to add CuSO4.5H2O as well as the use of D2O.

****We agree it would be good to include the rationale for this. We have added the reason for the use of copper sulfate to the protocol, which is to reduce T_1 and T_2 relaxation time, and the reason for the deuterium is to prevent radiation damping (lines 163-170)*

Line 155: Please add the reason of using 1g/L CuSO4.

**** The concentration is sufficient to cause strongly enhanced relaxation rates, but also stay below the saturation limit, thereby avoiding precipitation. This reasoning has been added to the protocol (lines 167-168).*

Line 161: Please write it down which specific vector network analyzer used in this experiment with its vendor details on table of materials.

****The ultilized vector network analyzer has been added to the table of materials.*

Line 161: Please specify the frequency range used in this method.

****The frequency range was 10 Mhz; this has been added to the text.*

Line 162: It would be again beneficial to write it down which S11 and Q-factor values are targeted for the VNA calibration step.

****We concur with the reviewer that target values could be helpful, but we are afraid they would be too specific for our system and also depend on the application of the coil. We would like to refrain from giving explicit design goals for this protocol, as the objective is more to give the reader a comprehensive overview of what to do with a new coil setup. Specific instructions for coil design and calibration can be found in Haase et al, which we would not like to replicate in this protocol.*

Line 167-169: What are reference and biological samples and how they are different? What are their roles?

**** The reference sample is meant to be used for coil characterisation, including the reference pulse power, volume normalised SNR and consist of the CuSO4 solution. The biological sample preparation is meant for general experimental use, this has been clarified in the text.*

Line 180: The microcoil holder is mentioned here for the first time. It would be nice to add it when the coil was described. Please add an explanation about its role.

****We have now introduced the purpose of the microcoil holder in the introduction (lines 104-105).*

Line 237: What is exactly the probe base? It is on the Micro5 probe?

**** The micro5 probe has an integrated gradient, unlike most probes. To differentiate between the whole probe including the gradient and the probe without the gradient, we use the term probe base when we mean the probe without the gradient. This may also be seen in Fig 3, showing the components of the probe.*

Line 247: Could the authors further explain the potential susceptibility problems due to the coil wires? Is the selection of the coil wire in this experiment avoiding this problem? If yes, please address how it has been done.

****We thank the reviewer for this question; the demonstrated coil uses fluid susceptibility matching, but using zero-susceptibility wire is also a viable option. The B_1 estimation included in the protocol can help to determine whether the effect is problematic enough to warrant the inclusion of susceptibility matching in the design of the coil assembly. We have expandend our thoughts on this in the discussion section (lines 600-610).*

Line 250: Could the authors be specific about the "all cables"?

****We have now included all the cables that need to be connected (line 279).*

Line 252-255: It is very beneficial to write which MRI system is used in this experiment (model/software version/vendor details/main magnetic field strength/type, etc/.)

****We agree that this would be useful, and have added into the introduction and protocol so that the reader will have a clear sense of the relevance for their own applications (lines 105-107).*

Line 264: from the line 135, only one type of capacitor looks like variable. Here the authors mentioned as two types of capacitors are variables. Could the authors make it clear?

****Indeed, the microcoil assembly only has one variable capacitor, which is set to the correct setting during the manufacture of the microcoil. We refer here to the tuning and matching capacitors found on the probe base, to which the microcoil assembly is mounted. We have now made it more clear in the text that we mean the probe base capacitors.*

Line 267: Please write it down the tune and match values at the desired frequency. It could be written in a range of targeted values.

****We would like to include the values, but the particular coil used here is hand-controlled use thumbscrews, and the values cannot be read accurately. These values are also very specific to the coil and even more so the sample. Instead, we recommend setting the tuning and matching to the middle of their ranges for new coils, this has now been added to the text (lines 306-308).*

Line 269: Please mention the targeted coil quality factor value for the described measurement method.

****Although we aimed for a Q-factor higher than 150 for our coils, it is very specific to the coil diameter we were using. Thus, we would preferably not include it as an explicit design goal for this protocol. We would also encourage an approach of optimizing for the volume normalized SNR instead of the Q-factor.*

Line 274: What does it mean "initiate a wobble curve"? Is the step 4.8 applied on VNA or the scanner?

****It was indeed performed on the scanner so that the reflectance test was performed in the actual experimental environment. We have added this information to step 4.8.*

Line 294: Please use the capital letters for RF.

****Corrected.*

Line 307: Is not it better to localize the sample first and then calibrate the RF power? In case the sample needs to be relocalised again, the RF power should be calibrated one more time. So, I suggest reordering the procedure for the step 4.13.

****We agree with the benefit of the proposed change, however the risk for damage to the coil is high for the first calibration, when the approximate power levels are unkown. For routine use, we certainly would change the order. We have added a note to explain the need to recalibrate the coil after repositioning.*

Line 327: What is the motivation of using spin-echo sequence for the SNR measurement rather than using GRE-type sequence? How sensitive to this experiment to the B1 homogeneity? It is good to give B1 homogeneity value for the same ROI used in the SNR measurement.

****We thank the reviewer for the interesting question. The spin-echo sequence is used to separate the SNR measurement from the B_0 inhomogeneity, and also lessens the variability due to B_1 inhomogeneity. The different number of slices can be used to measure the B_1 homogeneity and the linear range of the coil, using the reference sample.*

Line345: It is not clear to me where mu ROI (2.3) and noise (0.34) are calculated in the image. It could be good to give the dimension of ROI.

****We agree that more information on the ROI is beneficial. The exact size of the ROI is not critical as we are using mean values. The placement, however, is more important as the mean signal ROI should fall entirely within the sample and the noise ROI should be placed in the corners. We have added a new figure to show this (Figure 7).*

Line 370: Please write it down RF amplifier gain/RF input power used in the experiment.

****We used the automatic adjustments for most imaging, so we have included the recommended automatic adjustments in the protocol (419-422).*

Reviewer #2:

Manuscript Summary:
The paper describes a protocol how to image small biological samples using magnetic resonance microscopy at ultra-high field, achieving a best resolution of 13 microns. The entire protocol starts at the point, when the home-built microcoil is available. For the manufacturing of the microcoil, they refer to the literature. On the other hand, it is important that this type of coil has a good signal to noise ratio per volume and can be reproduced at low cost facilitating its broader use. The high magnetic field causes special problems due to potential susceptibility artefacts at air cavities and - interfaces arising around the rf-coil wire and inside the sample. They overcome this issue by submerging the coil and the sample into a susceptibility matching fluid.
The protocol consists of two parts. The first part contains a step-by-step sample preparation which is clearly illustrated by many photos. In this context, the author notes on chemical properties of the substances used are very useful. The second part of the paper deals with determination of the coil characteristics comprising the basic necessary steps. The authors describe all steps in high detail, supported by exemplary diagrams and MRI images. Additionally, reasonable notes and warnings complete the description. In summary, the protocol allows also non-experts to reproduce the procedure. Finally, the authors demonstrate the success by high-resolution images of a root and a root nodule by using different MRI pulse sequences.
Since I had no access to the author´s video, I cannot review its quality. The figure quality is good and the captions are detailed but not lengthy. Summarizing, the paper fulfills entirely the requirements of JoVE for publication after some minor corrections.

Major Concerns:
none

****We thank the reviewer for insightful comments and suggestions, are responses are found below.*

Minor Concerns:
Line 56: (13 micron)^3 yield a volume of 2.2 pL (picoliters).

****Indeed, thanks for spotting this, we have corrected the volume.*

Line 80: A section "Materials and Methods" does not exist.

****We have changed the sentence to point to the correct Materials list. (Line 80).*

Line 98: "… Calibrating the 90° pulse length or power…"

****Indeed, only the pulse length or power needs to be calibrated. We have rephrased the sentence as suggested by the reviewer.*

Line 118: Co-registration with optical microscopy is not included in this paper.

****We agree with the reviewer that co-registration is not covered with this protocol and have rephrased the sentence, so that only points out the possibility of combining it with this protocol.*

Line 127: Typo: delete last "is"

****It has been corrected.*

Line 142ff and Fig. 1B: The authors write that the sample was prepared in a 1mm sample capillary. It is not clear if this capillary in inserted in a second capillary, around which the coil wire is wound, or if the sample capillary is inserted directly into the solenoid. Further, it is not clear if the susceptibility matching liquid is filled into the coil compartment before, or after sample insertion. A schematic drawing of the setup with the different compartments would be helpful.

****We have moved the schematic drawing in figure 2 to Figure 1 so that the relationship becomes clear. The sample capillary is indeed inserted into the second capillary, which forms a permanent part of the microcoil assembly and the susceptibility matching fluid is added during coil construction.*

Line 155: Include the molarity of the reference solution and it´s relaxation times.

****We have added the molarity of the solution. Interestingly, it is not straightforward to measure the relaxation times of samples at ultra-high field, because traditional T_2 maps become highly resolution-dependent. Though, of course, whole sample T_1/T_2 can be determined, this is less relevant for imaging (line 168).*

Line 161: Describe briefly the "S11 test".

****We have added some lines to clarify the S_{11} test (line 172-184).*

Line 192ff: Many roots possess a rhizosheath, i.e. soil particles are strongly "glued" to the root and can hardly be removed without damaging the root. How did you deal with this issue?

****The growth substrate used consisted of relatively large grains of perlite, which could easily be identified by the naked eye and then selectively removed. Typically removing the particles with a pincer is sufficient, but washing in the sample in water is possible. Furthermore, sections of root without any visible rhizosheath were selected for measurement. We have added this clarification to the text (line 215-219).*

Please indicate the used root section: taproot or lateral, young, or old etc.

****We have added a sentence explaining the root section, which was five week old fibrous root (line 219).*

Line 201: How strong was the vacuum? Did you use a water jet or an oil pump?

****The pump used was a membrane vacuum pump, the type has been added to the list of materials and the protocol (line 226). The pump end vacuum pressure was 7 mbar. Vacuum was applied until no bubbles were seen escaping the sample, which we have now clarified in the text.*

Line 271: Isn´t it actually a reflection dip and not a resonance peak? f1, f2, and fc are not defined. A figure or printscreen of the network analyzer would be helpful.

****Indeed this would be a reflection dip, we have rephrased it accordingly and added a description of the variables to the text. It can, however, also indicate that there may be multiple resonant modes in the coil, as discussed later (lines 411-414).*

Line 317: Typo: delete first "pulse".

****It has been corrected.*

Lines 337ff: Equation 2 and description in the text do not match.

****We agree with the reviewer that the text could be more clear and have rewritten the paragraph accordingly (lines 381-386).*

Line 343 and Eq. 3: Is this the result for the reference solution?

****Correct, we have rephrased the sentence to make clear that the result of Eq. 3 are for the reference solution.*

Line 357: "longer" echo time, not "higher".

****It has been corrected.*

Line 365: The authors mentioned the existence of multiple resonance modes in the wobble curve. Please give more details about possible reasons.

****We have found that some microcoils, when tuned incorrectly, can resonate outside the actual coil, .e.g., near capacitors. A sentence explaining this has been added to the discussion (line 593).*

Line 368 ff or in the discussion: A FLASH or any other gradient echo sequence works well for the reference solution and biological samples with long T2 relaxation times. Please discuss that the reader should be aware, that other systems might possess shorter T2 relaxation times requiring spin echo sequences or even sequences with ultra-short detection times. This is typical for many materials and geological samples.

****We fully agree that this protocol would need to be adapted in order to work for samples with very short T_2 relaxation times, and have added a note to this effect to the discussion (lines 580-583).*

Line 369 ff: Please add the flip angle used. How do you avoid intensity loss due to saturation effects?

****The flip angle has been added to the text. We found that a flip angle of 30° gave an optimal result in terms of signal intensity per unit of time, we were aiming for optimal contrast and not a quantitative measurement, so we were not concerned with saturation effects.*

Line 389: Is this the calculated or the measured Q factor.

****We have added clarification in the sentence to indicate this is the measured Q-factor (Lines 388-389)*

Line 391: The value of 11 microseconds is different from the value given in figure caption 5, line 469 (12-13 microseconds).

****The correct value was 12 microseconds, we have removed the inconsistency in lines 391 and 469.*

Line 392: Tau and gamma are not defined.

****We have added tau and gamma to the text.*

Figure 1B, line 423: Please add the length of the coil and its inductance. The matching bath is not clearly visible, see comment on line 142, above.

****We have added the length of the coil to the text. As for the inductance, we have found it to be difficult to determine for solenoid microcoils this small so that no accurate measure can be given. To show the components of the microcoil more clearly, we have combined Figure 1 and 2 in a single figure. Now the schematic can be seen in relation to the photo of Figure 1B.*

Line 546: check grammar.

****It has been corrected.*

Reviewer #3:

Manuscript Summary:
The manuscript and video entitled "Magnetic Resonance Microscopy using Microcoils at 22 T: Coil Performance Calibration and Usage Demonstrated on Medicago truncatula Roots" by van Schadewijk et al. describe a procedure of preparation and optimization of MRM experiments of biological samples with a dedicated microcoil. The idea is to use microcoils adapted to the size of the sample.

The video is illustrative and shot in a professional way.

The associated manuscript is well written and its structure is clear. It looks to me already very smooth and close to a publishable form. Please find below a list of smaller mistakes and other points that should be corrected or clarified.

The content of the work is perhaps not absolutely novel (published in a different format in ref. 26), but its form of presentation gives a good insight into the procedure so that other researchers can learn from it.
I therefore recommend the publication of the work by JoVE.

****We thank the reviewer for the kind words and helpful comments.*

Minor Concerns:
l. 6/7 The formatting of the authors' list is messed up. For example, who is Aldrik, an author without first name, or only first name, without affiliation, ...?

****We thank the reviewer for pointing this out, a comma was indeed missing. It has been corrected.*

l. 71 "The SNR is inversely proportional to the coil diameter".
There's a bug in the video concerning this phrase.

**** The bug has been corrected, we thank the reviewer for noticing this.*

l. 81 "microcoils can be constructed using ..., an assortment of capacitors, ..."
The capacitor is not part of the coil.

****We agree that capacitors are not part of the coil, thus we have clarified the sentence to indicate we mean a complete microcoil assembly (line 87).*

Fig. 1B It is a bit difficult to identify the details of this figure, perhaps due to the image's quality and resolution. Labels within the figure would help.

****We agree the figure was hard to read. Therefore, we have combined figures to make more clear that Fig 2 (now Fig 1C) is the schematic representation of the coil in Fig 1B. We have also enlarged the image, together this should make it more easier to see the different components of the microcoil.*

l. 103 "suitable narrow-bore (≤ 60 mm) vertical spectrometer"
Should rather be the "magnet".

****We agree and have changed it to magnet.*

l. 107 "is housed is" Typo.

****It has been corrected.*

Fig. 2 As far as I understand, only the matching capacitor is variable, not the tuning one. How is the system tuned? Or is the band width of the circuit so large that tuning is not necessary? On the other hand, the video shows conventional tuning by probe capacitors. This point should be clarified.

****We thank the reviewer for pointing out this unclear section. In normal operation, the coil is tuned using the tuning and matching capacitors located in the probe-base. The variable capacitor on the microcoil itself allows for fine-tuning as in microcoils the tuning and matching is not completely orthogonal, Fine-tuning is perfomed only during manufacture of the coil. We have added some sentences explaining this further in the manuscript (line 102-104).*

l. 199 "Then, punch a hole in the with a sharp tool" Typo.

****It has been corrected.*

l. 146 "... which fits the Bruker Micro5 probe".
It should be mentioned already before that this type of commercial probe is used.

****We have added this information to the introduction at line 107.*

Fig. 6 12 µs for a p90 appear to me pretty long for a microcoil. The power, 0.6 W, is quite low. Is this due to the sensitivity of the small capacitors against high voltages?

****Indeed, the pulse power is deliberately kept low to avoid damage to the microcoil due to overvoltage. For this reason, we caution using high pulse power (Lines-321-324).*

Eq. 2 What is the exact definition of μnoise? Is this term necessary?

****Here, μ_{noise} stands for the mean signal of the noise. Though some definitions leave it out, we prefer it to keep it in order to account for anomalous signals, which may affect both the background and sample.*

l. 337 For how many repetitions is the SNR determined? One?

****That is correct; ideally, the SNR is determined on one repetition. We have added a clarifying sentence indicating this.*

l. 415 The legends are placed into the middle of the manuscript. (I guess this will be corrected anyway in the final version).

****That is correct, this follows the JoVE author guidelines.*

There are some errors in the reference list, e.g. references 4, 5, 11, 22

****Thank you for pointing this out, the references have been checked and adjusted. The DOI number of references 11 and 22, though odd, are correct.*