Extending electron paramagnetic resonance (EPR) spectroscopy to nanoliter volume protein single crystals

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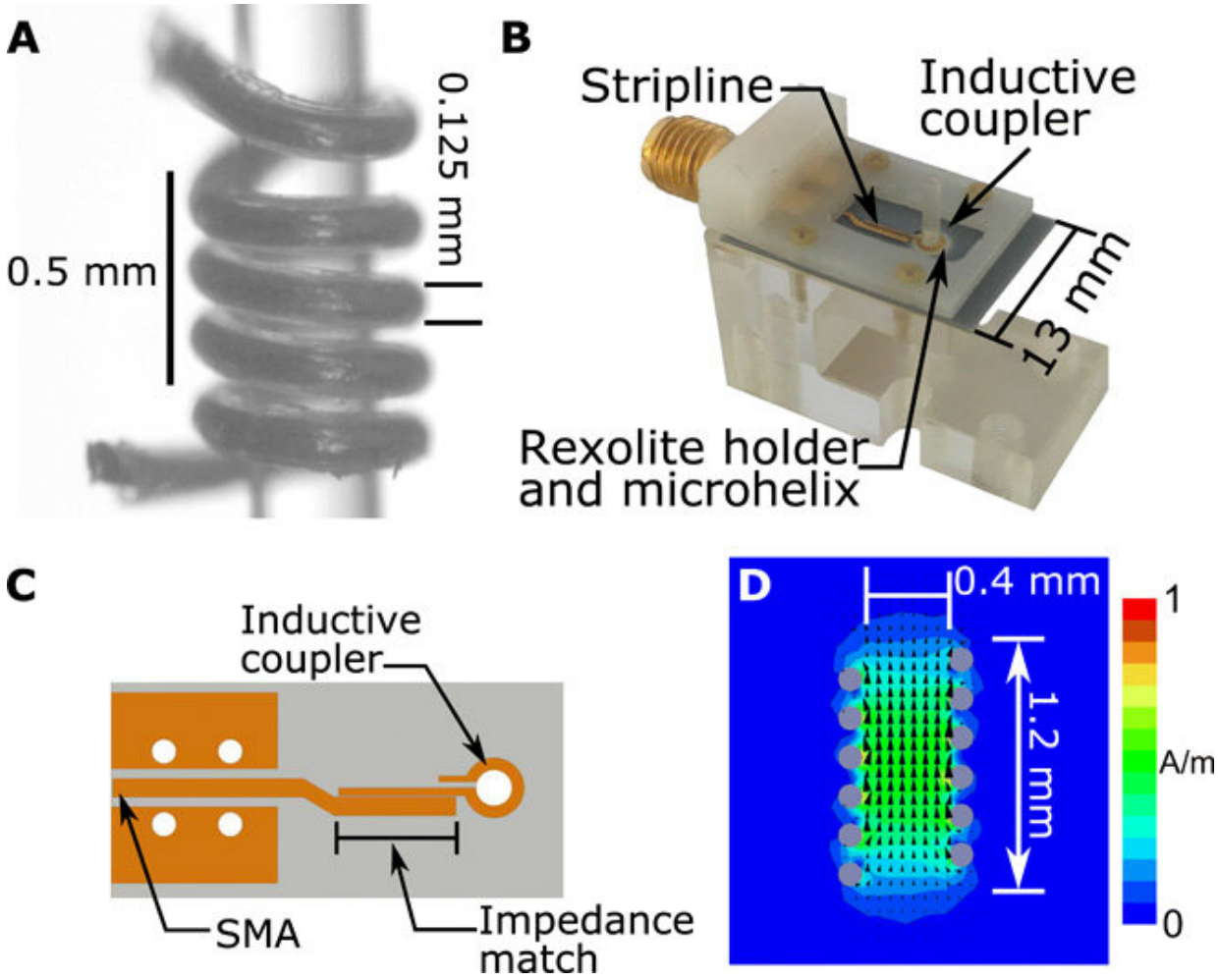


The self-resonant microhelix. (A) A fabricated five-turn microhelix wrapped around a 0.4-mm–outer diameter capillary. During fabrication, the microhelix is tightly wound around a 0.4-mm drill bit and glued inside a Rexolite cylinder. The

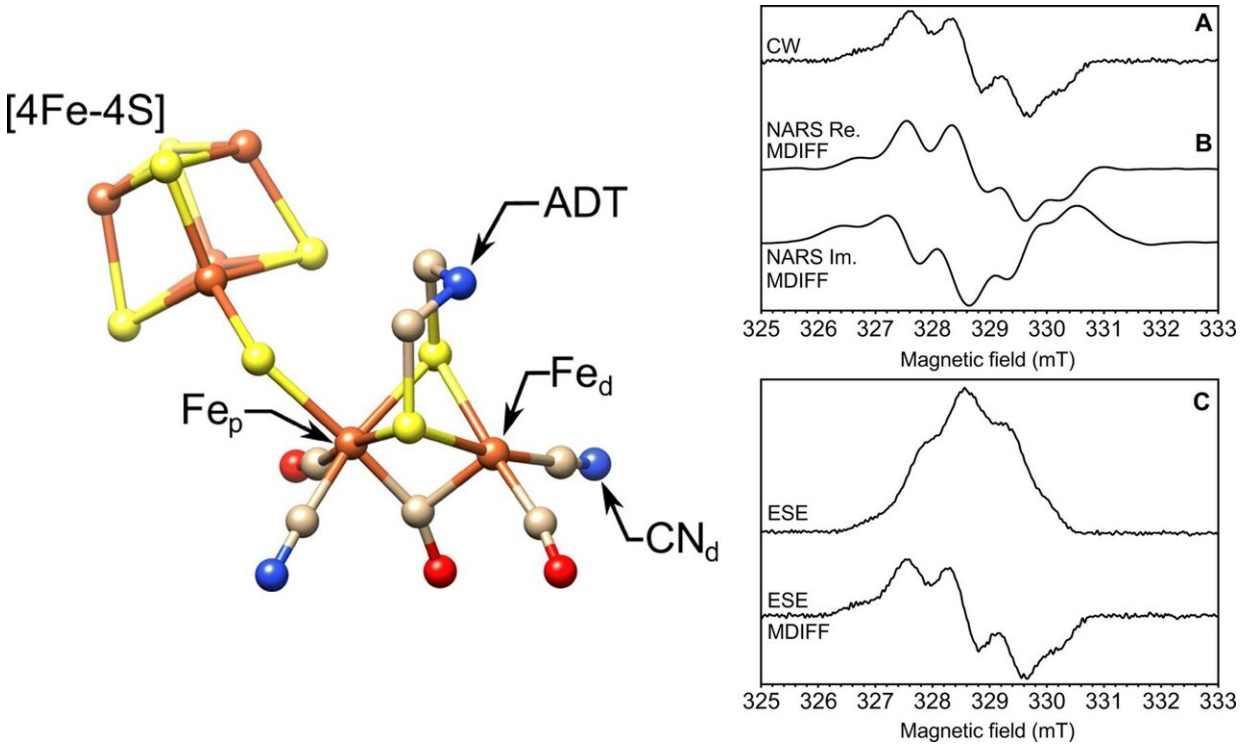
drill bit is removed, and the glue is allowed to dry for several days. The microhelix assembly is placed in (B) a coupling and support assembly, which includes a planar microcoupler. (C) The planar microcoupler consists of a stripline impedance match to an inductive coupling loop. SMA, Sub-Miniature version A. (D) Finite-element modeling simulations of the microwave magnetic field, normalized to input power, at 9.5 GHz show an active region of good magnetic field homogeneity over a 0.8-mm height. The measured microwave magnetic field of 3.2 G/W1/2 corresponds to a 20-ns π/2 pulse at approximately 20 mW. Dimensions of the microhelix, where the self-resonance is determined by the capacitance formed between each turn and the inductance of the windings, are shown. The frequency can be tuned during fabrication by the number of turns, the pitch of the turns, or the inner diameter. Credit: Science Advances, doi: 10.1126/sciadv.aay1394

Biochemists can use electron paramagnetic resonance (EPR) on protein single crystals to determine the ultimate electronic structure of paramagnetic protein intermediates and investigate the relative magnetic tensor to a molecular structure. The method is, however, withheld by typical protein crystal dimensions (0.05 to 0.3 mm) that do not provide sufficient signal intensity during protein crystallography. In a new study on *Science Advances*, Jason W. Sidabras and an interdisciplinary research team in the departments of Chemical Energy Conversion, Photobiotechnology, Institute for Biology and Experimental Physics in Germany presented a microwave self-resonant microhelix to quantify nanoliter samples. The scientists implemented the technique in a commercial X-band (mid-range frequency; 9.5 GHz) EPR spectrometer. The self-resonant microhelix provided a measured signal-to-noise improvement compared to other commercial EPR resonators. The work enables advanced EPR techniques to study protein single crystals for X-ray crystallography, without size-related exclusions or challenges. To demonstrate the method, Sidabras et al. used single crystal protein [FeFe]-hydrogenase (from *Clostridium pasteurianum*) with 0.3 mm by

0.1 mm by 0.1 mm dimensions.

Primary author Jason W. Sidabras, presently a Marie Sklowdowska-Curie Actions Fellow at the Max Planck Institute for Chemical Energy Conversion in Germany, further commented on the work conducted with fellow researchers Professor Wolfgang Lubitz and Dr. Edward J. Reijerse. "Although we started with [FeFe]-hydrogenase here, we have tried to investigate single-crystal EPR dynamics for years and the present technology isn't limited to transition metals alone. The method defined in the study is applicable to monitor any enzymatic activity within a stable protein intermediate." He further noted of their aim to use the technology to reduce existing costs of pulse EPR technology and replace costly high-power amplifiers for frugal science (economically cost-effective strategies in science).

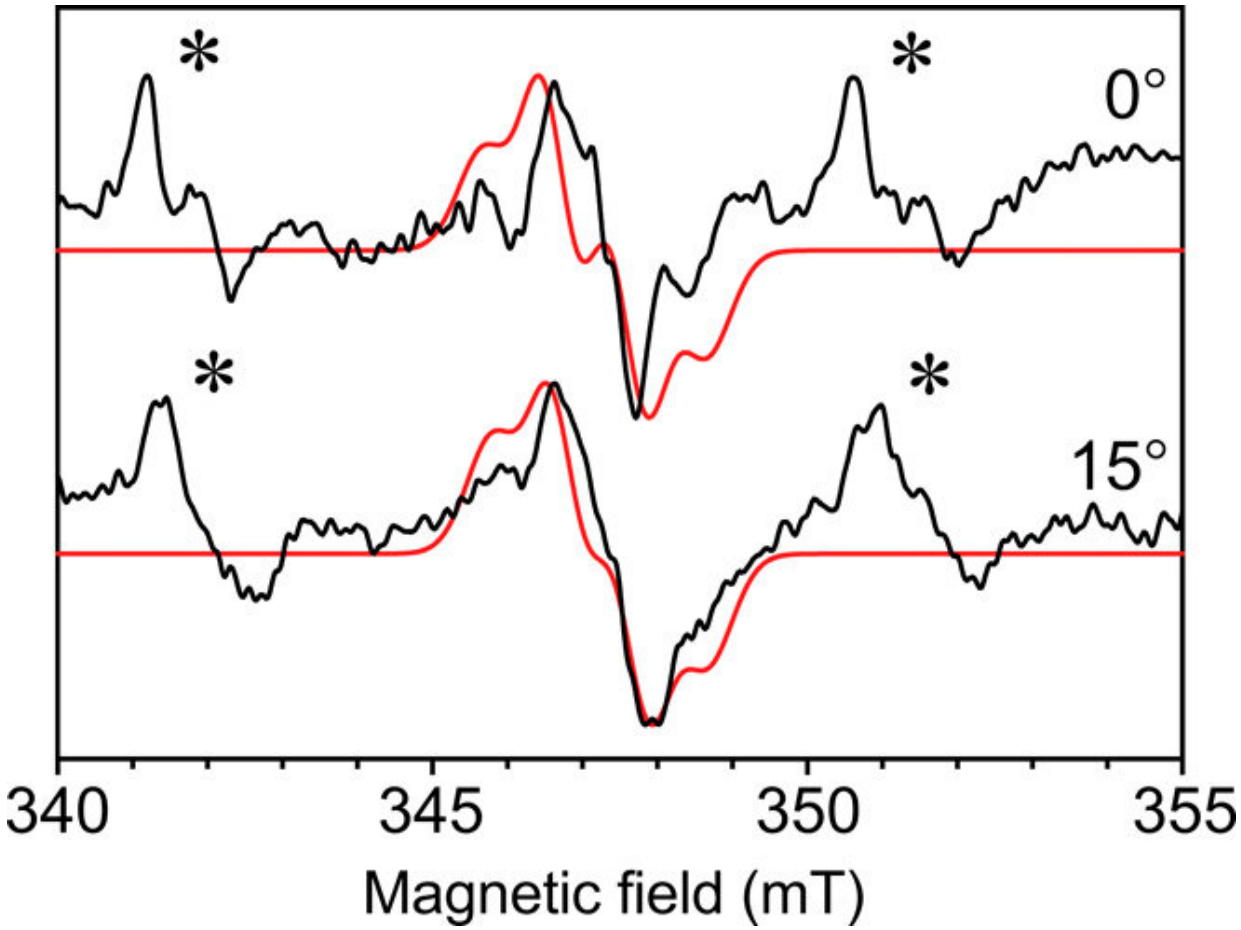
Scientists typically use EPR spectroscopy to investigate the catalytic cycle of redox enzymes that contain paramagnetic intermediates and obtain information on the electronic and geometric structure of an active enzymatic site. Generally, in order to conduct EPR experiments on proteins, researchers prepare a frozen solution (concentration between 0.1 to 1 mM) and place a volume (200 µl) in a microwave cavity to obtain magnetic interactions at an active site, with limited view of the electronic structure. To fully resolve the tensor magnetic interaction parameters, they must perform single-crystal EPR experiments where magnetic interaction tensors can be combined with X-ray crystallography to demonstrate protein geometry and understand catalytic mechanisms of enzymes. However, single-crystal EPR is rarely applied to protein systems due to challenges of obtaining crystals with appropriate volumes and sizes. Many proteins in the 0.05 to 0.3 mm range are too small for analysis using commercial EPR instrumentation.



LEFT: The molecular structure of the [FeFe]-hydrogenase active site, the H-cluster. Highlighted are the proximal and distal irons, Fep and Fed, respectively, the cyanide ligand (CN−d), and the ADT ligand. S, yellow; Fe, orange; N, blue; C, tan; O, red. Structure is from Protein Data Bank (PDB) ID 4XDC. RIGHT: Frozen solution EPR on an 85-nl-volume sample at X-band. Three EPR experiments performed with a 0.4 mm inner diameter self-resonant microhelix. Shown are the (A) continuous-wave (CW), (B) real (Re.) and imaginary (Im.) nonadiabatic rapid scan (NARS), and (C) field-swept two-pulse ESE EPR experiments of the tyrosine D radical (Y•D) in photosystem II with 85 nl of frozen solution sample at a temperature of 80 K. Calculated MDIFF (moving difference) pseudo-modulation of 0.5 mT is shown for the NARS and field-swept ESE experiments to directly compare to the continuous-wave EPR experiment. The total time for the experiments was 49, 55, and 45 min, respectively. The signal-to-noise ratio is calculated and tabulated. Credit: Science Advances, doi: 10.1126/sciadv.aay1394.

To improve the EPR sensitivity to study single crystals, typically at the X-band, researchers must abandon the microwave cavity design and move toward small-volume resonators in the microwave range. The strategy can facilitate reduced sample volumes from 200 to 20 µl using a loop-gap resonator (LGR) and additional reductions with high dielectric constant materials to reduce the active volume to one microliter. Protein single crystal investigations require even further volume reductions (less than 0.03 µl) and that calls for a radical approach. To accomplish this, Sidabras et al. combined a self-resonant microhelix and a planar microcoupler on a printed circuit board setup, which drove the self-resonant microhelix placed in the center of the coupling loop. The microhelix geometry offered advantages with a strongly improved microwave field homogeneity and higher volume sensitivity for small samples compared to other microresonators. The team optimized the self-resonant microhelix for pulse and continuous-wave experiments requiring very little microwave power. They easily matched and tuned the microhelix across a variety of samples and temperatures.

In the present work, the team used the self-resonant microhelix to investigate EPR crystal rotation of [FeFe]-hydrogenase in the active oxidized state (H_{OX}; crystal dimensions 3 mm by 0.1 mm by 0.1 mm), from *Clostridium pasteurianum* (anaerobic bacterium). They performed advanced pulse EPR experiments on the structure to observe excellent signal-to-noise ratio. The data demonstrated the use of the microhelix to study single crystal proteins at volumes appropriate for X-ray crystallography. During experiments, the research team wrapped the self-resonant microhelix geometry around a 0.4 mm capillary and attached the assembly to a custom insert compatible with commercial EPR systems. They conducted a continuous-wave EPR experiment using a frozen solution and improved the signal-to-noise ratio (SNR) of the work using a field-swept nonadiabatic rapid scan (NARS) experiment.

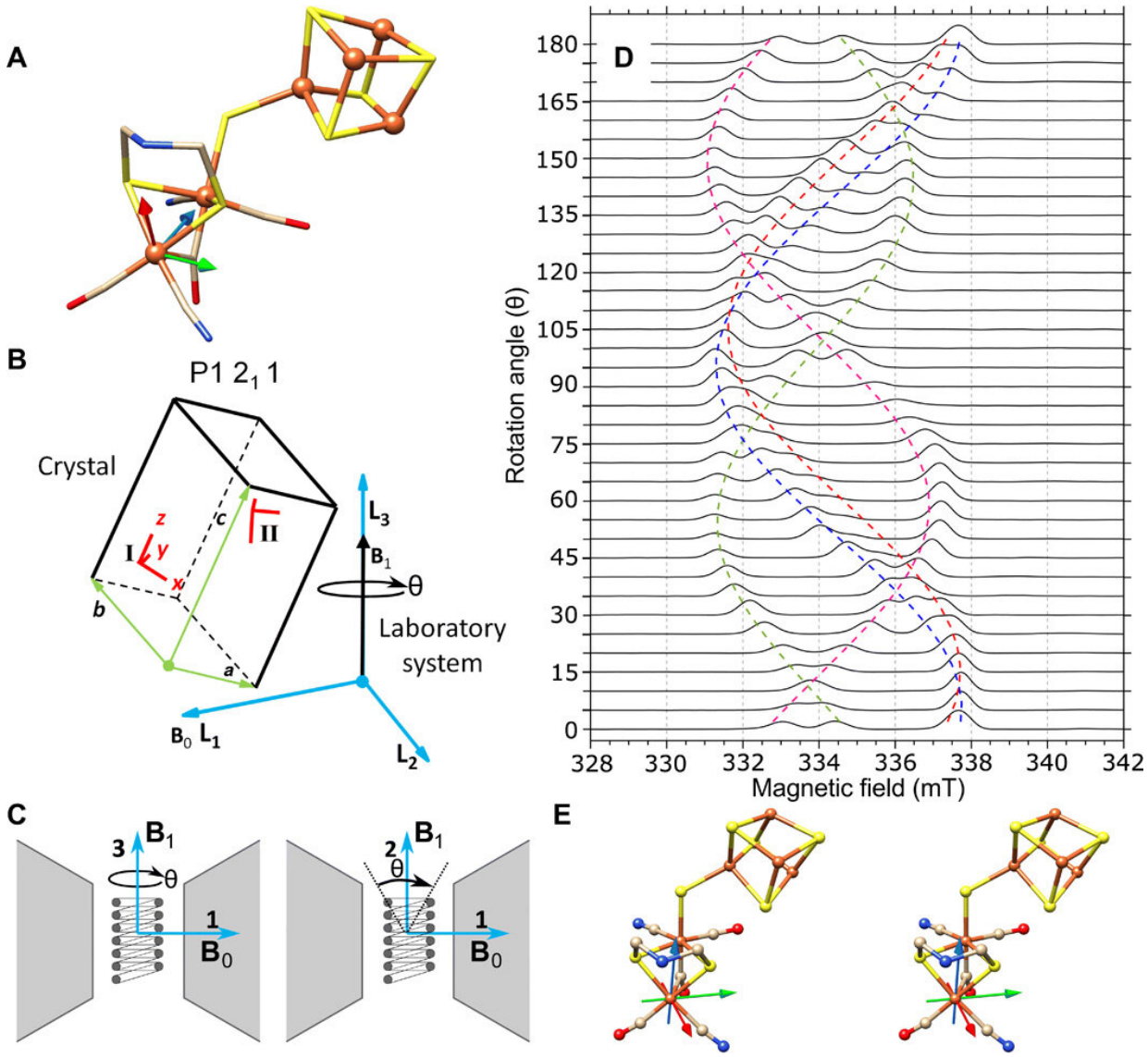


Single-crystal continuous-wave EPR of Y•D in the photosystem II core complex. Continuous-wave EPR collected with the 0.4 mm inner diameter self-resonant microhelix at two angles of the photosystem II Y•D radical from a single crystal at a temperature of 80 K. The crystal dimensions were 0.3 mm by 0.18 mm by 0.18 mm. Shown in red is a fitted simulation with similar features. A nonspecifically bound Mn2+ signal is also present in the mother liquor of the crystal, indicated by an asterisk (∗). Each spectrum was collected in 49 min with a signal-to-noise ratio of approximately 35. Credit: Science Advances, doi: 10.1126/sciadv.aay1394.

They used a long-lived tyrosine D radical (Y•D) as a standard probe during experiments with previously well-characterized properties. To

generate the tyrosine radical (Y•D) EPR signal, the team illuminated samples of the photosystem II core complex (membrane protein complex) in ambient light and rapidly froze them. They conducted multiple experiments to demonstrate versatility of the microhelix during EPR measurements across a variety of samples (less than 85 nanoliters in volume) at X-band. Sidabras et al. used the photosystem II crystals as a benchmark despite its challenging constitution. Structurally, the photosystem II complex contained a molecular mass approximating 350 kDa with each component containing only one Y•D radical. In total, with eight photosystem II complexes per unit cell the scientists calculated 8.9 x 10^{12}Y•D radicals, to demonstrate the versatility of the EPR method to study large complexes in small crystal dimensions.

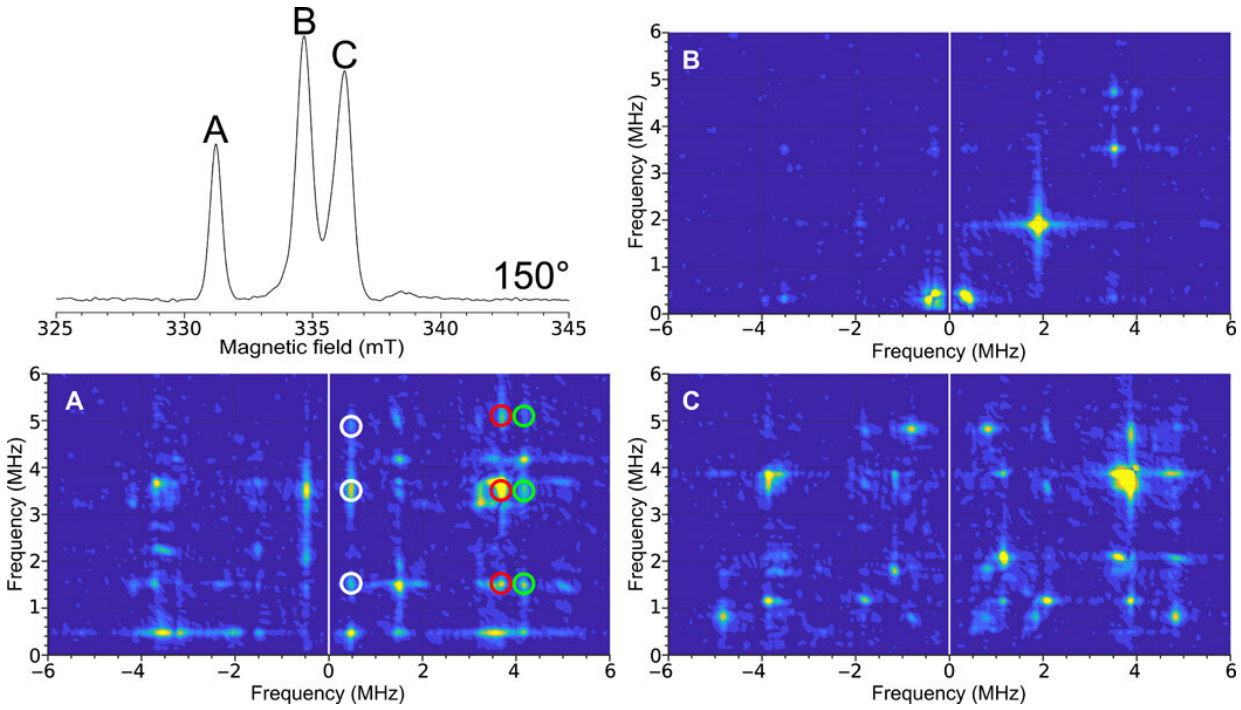
After establishing suitability of the self-resonant microhelix to study single-crystal protein samples, the team extended the work to demonstrate full angular g-tensor determination (energy shift associated with molecular transition) and to examine advanced pulse EPR experiments such as electron spin echo envelope modulation (ESEEM) or hyperfine sublevel correlation (HYSCORE). They optimized the self-resonant microhelix for these experiments. The team field-swept two-pulse ESE (electron spin echo) EPR experiments on a protein single crystal of the [FeFe]-hydrogenase of *C. pasteurianum* (Cpl) in the oxidized H_{OX} state in an anerobic chamber under a microscope to take up protein crystals via capillary action into a capillary tube.



Pulse EPR on a single crystal of the H-cluster in [FeFe]-hydrogenase. (A) The molecular structure of the [FeFe]-hydrogenase active site, the H-cluster, from PDB ID 4XDC is shown with the molecular frame located with the distal iron (Fed) as the origin. S, yellow; Fe, orange; N, blue; C, tan; O, red. (B) The P1211 symmetry schematic relating the molecular frame (x, y, z) to the crystal frame (a, b, c) and, last, to the laboratory system frame (L1, L2, L3) is shown. The two molecular frames from the asymmetric unit are present in Site I and can be translated to Site II by crystal symmetry operations. (C) The static magnetic field (B0) is positioned along the L1 axis, while the microwave magnetic field (B1) can be either along the L2 axis or along the L3 axis. A rotation of 180° is feasible around the L3 axis, but only a partial rotation around the L2 axis is

feasible because of the B1 rotating with the crystal resulting in B1 to become parallel to B0. A third partial rotation is feasible if the sample is rotated by 90° around the L2 axis. (D) Pulse EPR experiments collected with the 0.4 mm inner diameter self-resonant microhelix with a [FeFe]-hydrogenase single crystal of C. pasteurianum (CpI) in the Hox state showing collected data in one plane for a full rotation of 180° in 5° steps at a temperature of 15 K. The crystal dimensions were approximately 0.3 mm by 0.1 mm by 0.1 mm, and each spectrum was collected in 8 min with a signal-to-noise ratio of approximately 290. (E) A stereo view of the analyzed g-tensor (gx, red; gy, green; and gz, blue) is mapped on the crystal structure (PDB ID: 4XDC). For a three-dimensional (3D) view of the proposed g-tensor, see https://act-epr.org/FeFeHydrogenase.html. Credit: Science Advances, doi: 10.1126/sciadv.aay1394.

They then included cryoprotectant and media in the microhelix followed by flash-freezing to produce an EPR signal with four distinct signals in the spectrum relative to the protein structure. The scientists fitted the data into simulations relating to different frames of reference defined via the EasySpin simulation package for EPR spectrum simulation. The team created a schematic relating the [FeFe]-hydrogenase H-cluster molecular frame to the laboratory system frame. For all species examined in the experiments, the team determined the g-tensor magnitude and orientation using ligand-field theory and verified the results using quantum chemical calculations. The team facilitated fundamental insights into the electronic structure and noted their dependence on the ligand sphere and observed the necessity for optimized strategies.



Single-crystal HYSCORE EPR of the H-cluster in [FeFe]-hydrogenase. Top left: Field-swept two-pulse ESE EPR spectrum at 150°. The figure labels (A, B, and C) are representative of the spectral peaks. The HYSCORE spectra collected with the 0.4 mm inner diameter self-resonant microhelix of a [FeFe]-hydrogenase single crystal of C. pasteurianum (CpI) in the Hox state at an orientation of 150° collected at a temperature of 15 K. The 2D density representation shows correlations between the nuclear spin transitions in both projections of the electronic spin. (A) Clean HYSCORE spectrum due to the peak corresponding to only one of the EPR signals in the unit cell of the crystal. The correlated features between these transitions are indicated by the white, red, and green circles. (B) Relatively featureless HYSCORE spectrum suggests little hyperfine interaction at this orientation. (C) HYSCORE on two overlapping EPR signals representing different orientations of the enzyme molecule with respect to the magnetic field. The HYSCORE was set up using the Bruker HYSCORE wizard with the following settings: $\pi/2$, 40 ns; τ, 280 ns; and $\Delta\tau$, 48 ns with 256 points each and 20 shots per point. Each HYSCORE spectrum was collected in approximately 1 hour. Credit: Science Advances, doi: 10.1126/sciadv.aay1394.

The researchers illustrated more advanced experiments for single-crystal studies using HYSCORE (hyperfine sublevel correlation) experiments for the ESE (electron spin echo) EPR dataset. For this, they obtained a single-crystal 2-D spectrum for the H-cluster in [FeFe]-hydrogenase crystals and identified six main transitions. Sidabras et al. highlighted the feasibility of these advanced EPR techniques in the present work and related them to the electronic structure predicted using quantum chemical calculations. The team aim to address additional molecular couplings of ligands in depth using ESEEM/HYSCORE techniques in the future.

In this way, Jason W. Sidabras and colleagues presented an advanced resonator to design and collect EPR data from a 3 mm by 0.1 mm by 0.1 mm single crystal of [FeFe]-hydrogenase in the H_{OX} state from *C. pasteurianum* (Cpl). The HYSCORE spectra obtained from a protein single crystal in the present work were a first in study. Additional work proposed by the team will facilitate further insight for protein engineering and artificial enzyme research to create bioinspired and biomimetic enzymatic systems. Notably, the self-resonant microhelix engineered in the work can allow biochemists to study diverse catalytically active proteins at crystal dimensions relative to X-ray crystallography, which will pave the way for significant advancements in the field of enzyme research.

More information: Jason W. Sidabras et al. Extending electron paramagnetic resonance to nanoliter volume protein single crystals using a self-resonant microhelix, *Science Advances* (2019). DOI: 10.1126/sciadv.aay1394

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