Workflow for Setting up a DQC Experiment

The following protocol describes the DQC experiment on trityl-labelled protein samples using a Bruker (Bruker BioSpin, Rheinstetten, Germany) ELEXSYS E580 pulsed EPR spectrometer operated at Q-band with an ER5106QT2 resonator and a 150 W TWT amplifier (model 187 Ka, Applied Systems Engineering, Fort Worth, USA). For details on how to operate the spectrometer and the Xepr software, refer to the instrument manual and references [1] and [2].

Step 1 Power up the spectrometer and cool down the cryostat to a temperature of 70 K using a constant flow of cold helium gas. The temperature of 70 K provides a good trade-off between a long phase-memory time T_M and a short shot-repetition time (SRT).

Step 2 Insert the EPR sample tube into the sample rod so that the centre of the sample is located in the EPR-active zone of the resonator. For resonator-specific details, refer to the user manual of the spectrometer. Place the EPR sample tube in the resonator and over-couple the cavity as described in references [1] and [2].

Step 3 Make sure that the temperature is stable at 70 K and does not fluctuate (± 0.1 K). Wait at least 20 minutes until the temperature of the sample has stabilized before you continue. The resonator and the sample need to be thermally well-equilibrated before starting the measurements since temperature changes can result in phase and frequency instabilities. As trityl radicals have a narrow EPR spectrum, phase and frequency drifts can lead to a tremendous decrease in sensitivity.

Step 4 Perform the safety test of the detection system as described in the spectrometer manual and switch the TWT amplifier into Operate mode.

Step 5 Set the magnetic field B_0 to the value that corresponds to $g \approx 2.0038$ at the given MW frequency ν (e.g. B_0 = 12016 G at ν = 33.7 GHz).

Step 6 Program the Hahn echo sequence π/2–τ–π–τ–Echo using the pulse tables. Set π/2 = 12 ns and π = 24 ns as a first guess, use an interpulse delay of τ = 200 ns, an SRT of 6.5 ms, and accumulate 10 shots per point. Start SpecJet to monitor the Hahn echo and lower the MW attenuation to maximize the echo intensity, i.e. to obtain π/2- and π-pulses at the given pulse lengths. In our hands, π/2- and π-pulses are obtained at ~3-5 dB attenuation when using pulse lengths of 12 ns and 24 ns for π/2 and π, respectively.
Alternatively, set the MW attenuation to 0 dB and determine the optimal pulse lengths by a transient nutation experiment.

Step 7 Optimize the phase of the MW pulses such that the entire signal will be detected in the real channel of the quadrature detector. Slightly changing the magnetic field (usually < 3 G) may be helpful in this step to fully bring the sample on resonance. Optimize the video gain amplification such that the echo fills the entire SpecJet display without clipping; set the number of averages in SpecJet to 1 for this purpose. Check again the attenuation and make sure that the echo is still maximized.

Step 8 Record the echo-detected field-swept EPR spectrum. To this end, set the integration gate symmetrically around the echo maximum and adjust the gate width such that the entire echo is covered. For the field-swept EPR spectrum, integrating the whole echo is crucial to obtain a sufficiently high field resolution.[3] Set the sweep width to 200 G, the number of points on the abscissa to 400, and start the experiment. As the experiment runs, adjust the number of scans to be accumulated; depending on the spin concentration, a few scans (e.g. n = 3) should be sufficient to obtain a good SNR. Save the spectrum and read off the magnetic field value that yields the maximum signal intensity. Set the centre field to this value.

Step 9 Perform the inversion recovery (IR, π_{inv}–T–π/2–τ–π–τ–Echo) experiment as described in the instrument manual to determine the longitudinal electron spin relaxation time. In the IR experiment, the Interval T is incremented and the echo amplitude is monitored as a function of T. Running the IR experiment from PulseSPEL, the following values proved to be appropriate for trityls at the temperature of 70 K: π/2 = 12 ns, π = 24 ns, τ = 200 ns, T = 400 ns, ΔT = 80 µs, SRT = 70,000 * srtu, h = 1, 626 points on the trace. Read off the time when the signal intensity has recovered to ~80% of its maximal value; this yields an appropriate value for the SRT.

If set up correctly, the IR trace should smoothly transition into a plateau (Figure 1a, at T > 20 ms). However, if the trace shows a kink and abruptly transitions into the plateau (Figure 1b), the detector is saturated and the signal is clipped. In this case, reduce the video gain amplification.

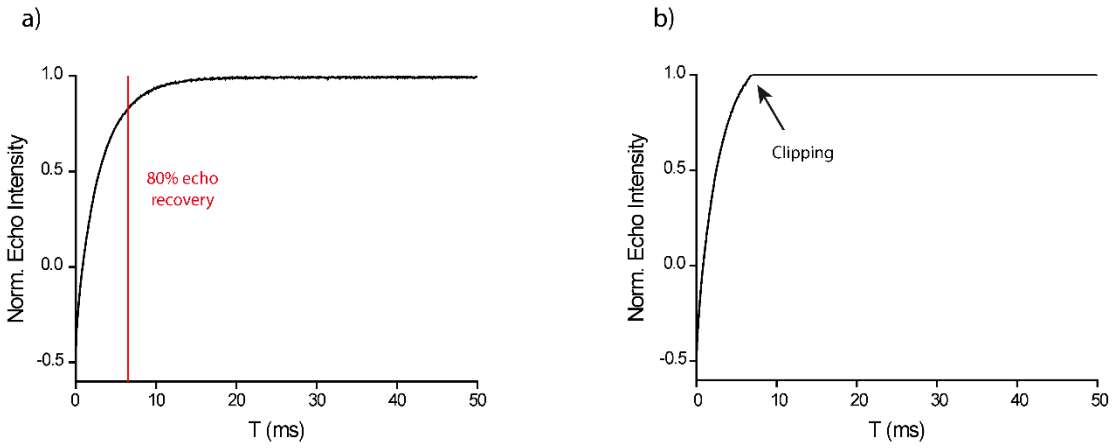

Figure 1: IR traces recorded with different video gain settings. a) 18 dB video gain, no clipping; the red line marks the value T = 6.5 ms when the echo has recovered to ~80% of its maximal intensity. b) 21 dB video gain, clipping can be seen from the absence of noise in the horizontal part of the trace and the kink indicated by the arrow.

Step 10 Perform the two-pulse electron spin echo envelope modulation (two-pulse ESEEM) experiment to obtain information on transverse electron spin relaxation. This experiment monitors the Hahn echo decay upon increasing the interpulse delay τ and permits inferring the maximally feasible dipolar evolution time in the DQC sequence. As detailed in the instrument manual, the two-pulse ESEEM experiment can be run conveniently from PulseSPEL using the following parameters: π/2 = 12 ns, π = 24 ns, τ = 200 ns, Δ τ = 8 ns, SRT = 6500 * srtu, h = 10, 2048-4096 points on the trace, depending on how quickly the echo decays (Figure 2).

Read off the time when the echo intensity has vanished to ≤ 5% of the initial amplitude. Note that the PulseSPEL program by default records the Hahn echo decay as a function of τ, whereas it is usually shown as a function of 2τ in the literature.[5]

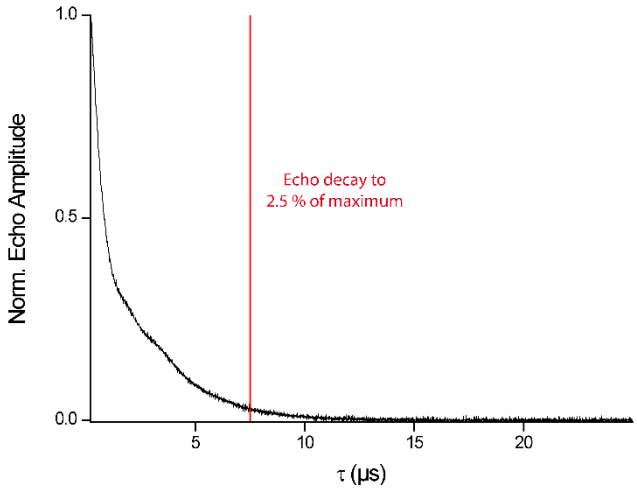

Figure 2: Hahn echo decay curve. Here, a dipolar evolution window of 7.5 µs (2.5 % of the maximal echo amplitude, marked by the red line) would be appropriate for the DQC experiment.

Step 11 Load the PulseSPEL file for the DQC experiment,[1] which contains programs for the standing Hahn echo ("2P ESE Setup"), the standing DQC echo ("DQ ESE Setup"), and the DQC sequence ("ESE DQ-EPR"). Also, load the variable definition file,[1] which sets the acquisition parameters (pulse lengths, delays, etc.), into PulseSPEL. Figure 3 shows the DQC pulse sequence and Table 1 lists the parameters of the DQC experiment with their conventional names and the corresponding PulseSPEL variables. Suggestions on values for the parameters are provided as well; however, one should always set the parameters (e.g. pulse lengths, SRT, trace length, etc.) to the optimal values determined in the previous steps. The meaning of the PulseSPEL variables d4 and d30 and the pulse timing of the DQC sequence are described in further detail in step 13.

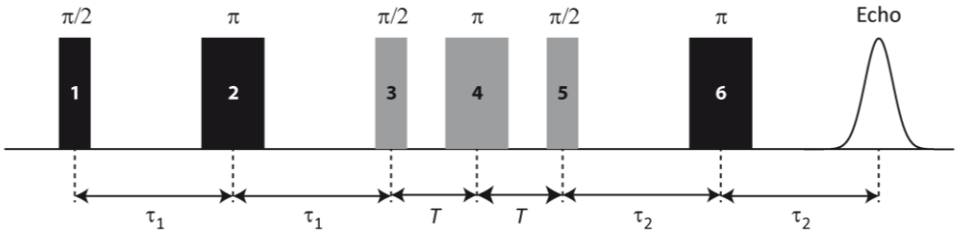

Figure 3: Scheme of the DQC pulse sequence. In the DQC experiment, the interpulse delays τ_1 and τ_2 are incremented and decremented by Δt, respectively, and the echo intensity is recorded as a function of $\tau_1 - \tau_2$.

[1] Can be found at https://github.com/TobiasHett/DQC_PulseSPEL

Table 1: Parameters for the DQC experiment.

Conventional Variable	PulseSPEL Variable	Typical Value / Comment
π/2-pulse	p0	12 ns
π-pulse	p1	24 ns
Initial value of interpulse delay τ_1	d1	200 ns, must be longer than the spectrometer dead time
Initial value of interpulse delay τ_2	d2	d2 = d1 + d4
Interpulse delay T	d3	50 ns
–	d4	Time to start before axis = 0, equals the desired trace length
Increment Δt/2	d30	Even numbers between e.g. 2 ns and 8 ns, depending on the period of the dipolar oscillation to be resolved. The increment on the time trace will be Δt.
Increment for nuclear modulation averaging	d31	16 ns (to suppress ^{2}H-ESEEM)
Steps for nuclear modulation averaging	m	8 (to suppress ^{2}H-ESEEM)
Shot repetition time	SRT	e.g. 6500 * 1.02 µs
Number of shots per point	h	e.g. 3
Number of scans	n	n ≥ 1

Step 12 Select the "2P ESE Setup" experiment from the dropdown menu, select the "+x none" phase cycling option, and monitor the Hahn echo in SpecJet. Note that only the variables p0, p1, d1, and SRT are relevant for the Hahn echo. Check the phase settings. To attain maximum efficiency of the phase cycle in the DQC experiment, careful adjustment of the MW phase is crucial, which can be done at the stage of the Hahn echo. If the MW phase has been adjusted properly at step 7, summing the Hahn echo obtained from $\pi/2_{+x}$ / π_{+x} and $\pi/2_{-x}$ / π_{-x}-pulses should cancel the signal in the real and the imaginary channel of the quadrature detector. The same holds for $\pi/2_{+y}$ / π_{+y} and $\pi/2_{-y}$ / π_{-y}-pulses.

The "2P ESE Setup" experiment with the phase cycling options "sumx" and "sumy" records the Hahn echo with the respective phase settings (+x/-x or +y/-y), sums up the echoes, and thus permits checking proper phase adjustment (Figure 4). If a substantial signal remains in the real or imaginary channel with either of the phase cycling options, consider (usually slight) adjustments of the MW phase.

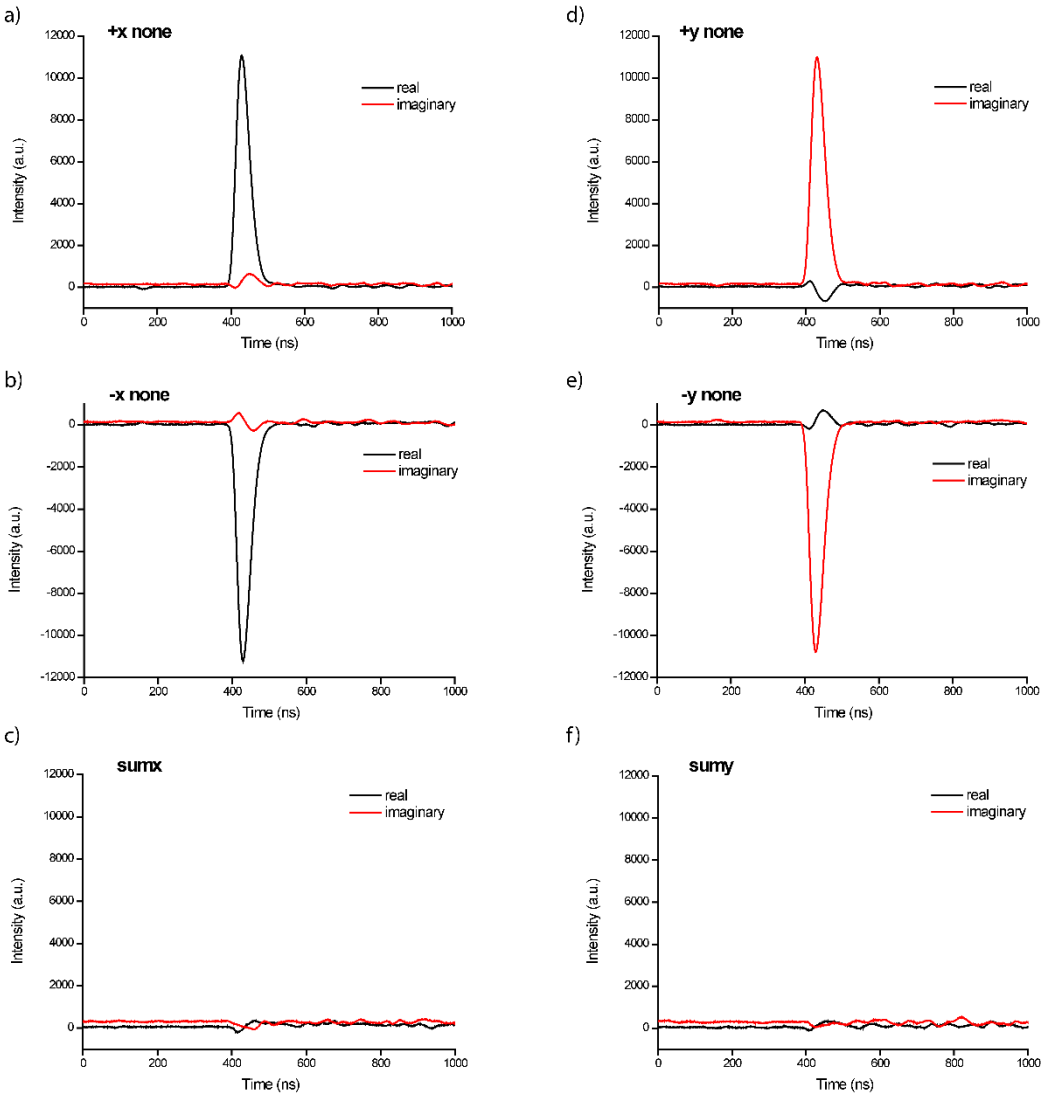

Figure 4: Hahn echoes recorded with different phase cycling options. a,b) Recording the Hahn echo with phase settings "+x none" and "–x none" inverts the signal by 180°, with "none" referring to the fact that no phase cycle is executed. c) Summing the signals from (a) and (b) with the phase cycle option "sumx" cancels the echo if the phase has been adjusted correctly. d-f) Analogous set-up experiment as in (a-c) with y-phase.

Once the MW phase has been adjusted, **do not change it anymore**. Imbalances in the phase will decrease the efficiency of the phase cycle in the DQC experiment and thus lead to artefacts in the time trace.

Step 13 For the following steps of setting up the DQC experiment, it is crucial to understand the timing conventions of the PulseSPEL program. The DQC trace is recorded as a function of τ_1–τ_2. At the start of the program, the interpulse delay τ_2 is set to the value τ_1 + d4, where d4 determines the length of the time trace. Upon integrating the DQC echo obtained with τ_1 and $\tau_2 = \tau_1$ + d4 (shown at the x-value τ_1–τ_2 = –d4, i.e. as the leftmost point on the trace), τ_1 and τ_2 are incremented and decremented, respectively, by the step d30. The next time point on the trace is thus τ_1–$\tau_2 = (\tau_1 + d30) – (\tau_1 + d4 – d30) = –d4 + 2d30$. This is the reason why the time step on the DQC trace ($\Delta t = 2d30$) is doubled compared to the setting d30 in PulseSPEL. Continuing this incrementation and decrementation scheme leads to a time trace symmetric about the maximum at the zero-time, i.e. when the condition $\tau_1 = \tau_2$ is fulfilled (Figure 5).

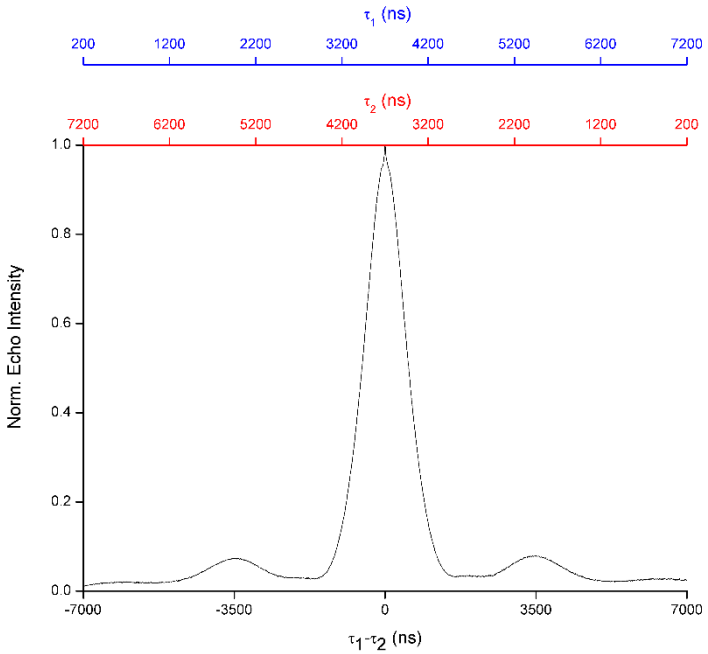

Figure 5: DQC time trace obtained with the interpulse delay parameters d1 = 200 ns, d2 = 200 ns, d3 = 50 ns, d4 = 7000 ns. The three x-axes show (from top to bottom) the interpulse delays τ_1 (blue), τ_2 (red), and the common representation of the trace as a function of τ_1–τ_2 (black). The DQC trace peaks at $\tau_1 = \tau_2 = 3700$ ns. Of note, the temporal position of the DQC echo does not change, as the sum of τ_1 and τ_2 is the same at every point on the trace ($\tau_1 + \tau_2 = 7400$ ns in this example).

Step 14 Decide on the length of the DQC trace (parameter d4). On the one hand, the maximum feasible length is governed by the echo decay observed in the two-pulse ESEEM experiment (step 10). On the other hand, at least two oscillations corresponding to the most probable distance in the distribution have to be resolved for reliable data analysis.[4,5] If there is prior knowledge on the expected interspin distance, calculate the oscillation period and thus the required trace length. Otherwise, determine a feasible value for the trace length from the Hahn echo decay curve (step 10). If you later realize that a longer DQC time trace is required to observe oscillations, abort the scan, increase the trace length, and restart the experiment. With the delay d1 and the trace length d4 for the DQC run, calculate the interpulse delays that fulfil the condition $\tau_1 = \tau_2$, i.e. that yield the maximum of the DQC time trace:

$$\tau_1 = \tau_2 = \frac{d4}{2} + d1 \qquad (1)$$

At this point of maximal echo intensity, the video gain amplification has to be adjusted such that the echo amplitude is maximized without clipping. Table 2 exemplarily summarizes the settings for the DQC run and the corresponding settings for the DQC setup.

Table 2: Exemplary interpulse delay settings for the DQC run and the DQC setup experiment.

DQC Run	DQC Setup
d0 = 404 ns	d0 = 0 ns
d1 = 200 ns	d1 = 3700 ns
d2 = d1 = 200 ns	d2 = d1 = 3700 ns
d3 = 50 ns	d3 = 50 ns
d4 = 7000 ns	d4 = 0 ns

Step 15 Set the values $\tau_1 = \tau_2$ calculated at step 14 for d1 and d2; set d4 = 0 ns, and the number of transient averages a = 1. Run the “DQ ESE Setup” experiment from PulseSPEL with the phase

cycling option “+x none”. Start SpecJet to observe the echo and preliminarily adjust the video gain such that no clipping occurs. The intensity of the DQC echo varies depending on the individual phase cycle step; this implies that the signal should be checked for clipping at each of the 64 steps of the phase cycle. To this end, deactivate the “On-Board Phase Cycling” option in the Xepr software (FT-EPR-Parameters → Options → On-Board Phase Cycling), select the “64-step” phase cycle from the dropdown list, and press the run button. Each of the 64 separate phase cycling steps (Figure 6) will now be executed and the respective echo will show up in SpecJet so that clipping can be checked for.

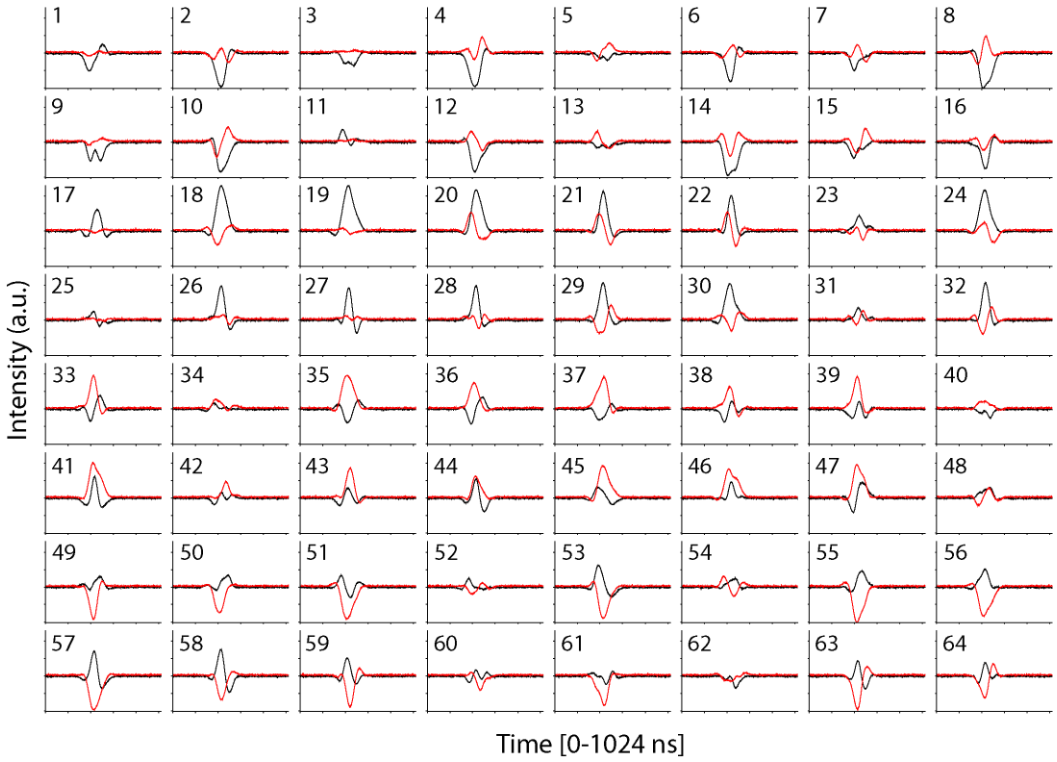

Figure 6: Representation of the individual 64 DQC echoes in the 64-step phase cycling procedure (see supplementary information of reference [6]). The black traces show the real channel of the quadrature detector and the red traces the imaginary channel. Note the different echo intensities depending on the step of the phase cycle.

Step 16 If clipping occurs at any of the phase cycling steps, decrease the video gain amplification, abort the experiment, and run it again. If the program execution crashes with an error message (“Time out while waiting for data from SpecJet II”), restart it. Note that the filtered DQC echo cannot be shown directly in SpecJet as it requires the 64-step phase cycle. However, after recording all phase cycling steps, the DQC echo will be shown in the viewport of Xepr.

Clipping has to be prevented as it leads to distortion of the DQC echo, which manifests itself in the broadening and splitting of the echo when summing up all 64 phase-cycling steps (Figure 7). Properly setting the video gain and optimizing the echo is thus crucial for unbiased measurements.

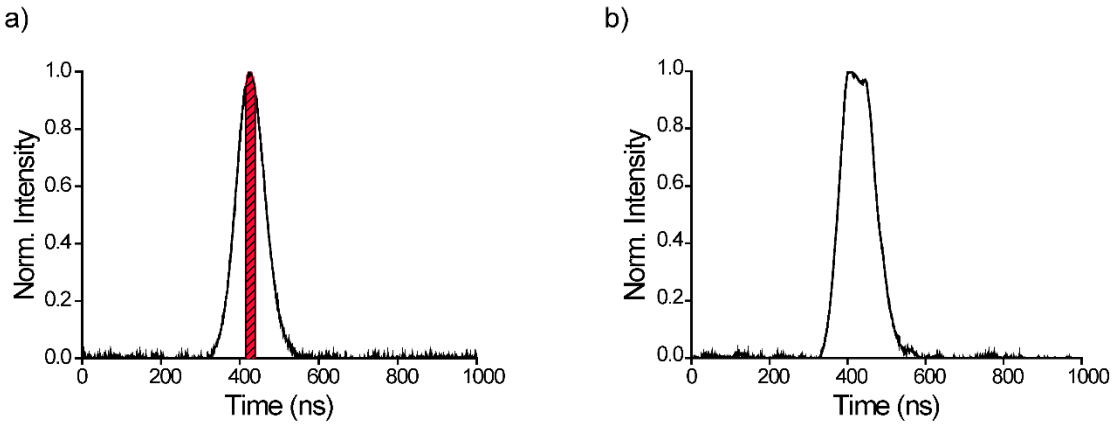

Figure 7: The DQC echo recorded at different settings of video gain amplification. The signal intensity has been normalized. a) 12 dB video gain, no clipping. The red area indicates the integration gate of 24 ns length around the echo maximum. b) 21 dB video gain, clipping. Note the broadening and distortion of the echo.

Step 17 Re-activate the "On-Board Phase Cycling" option. Press the run button to record the DQC echo again with the 64-step phase cycle and read off the position of the echo maximum on the time axis. Set the integrator gate width (variable pg in PulseSPEL) to the length of the longest pulse in the sequence and adjust the acquisition trigger offset (variable d0 in PulseSPEL) such that the echo is centred within the acquisition gate.[3] This maximizes the SNR of the DQC trace.

Step 18 Decide on the time step Δt on the DQC trace: On the one hand, the time step should be chosen small enough to allow for a sufficient resolution of the dipolar oscillations. On the other hand, setting the time step too small will unnecessarily increase the acquisition time without providing additional information. Depending on the oscillation period, time steps of Δt = 4 ns to 16 ns are most common; especially in the case of short interspin distances, the time step should not exceed 4 ns to resolve the steep initial decay. Note that for the reasons discussed at point 13, the increment d30 to be set in PulseSPEL equals Δt/2.

Step 19 Set the parameters d1, d2, d4, and d30 for the DQC run (Table 2). Calculate the number of points (NoP) to be recorded on the trace using eq. (2):

$$NoP = \frac{d4}{d30} + 1 \tag{2}$$

Set this value as the dimension of the abscissa for the "ESE DQ-EPR" experiment (dim2) in the PulseSPEL program.

Step 20 Select the "ESE DQ-EPR" experiment with the 64-step phase cycle from the dropdown list and press the run button. The spectrometer will now perform the DQC experiment including the phase cycling and nuclear modulation averaging procedures. The approximate runtime can be computed by

$$Runtime = h \cdot m \cdot n \cdot NoP \cdot PC \cdot SRT \tag{3}$$

Note that this is to be understood as a lower limit of the acquisition time, as it does not include the overhead of the pulse programmer.[7] The actual runtime can thus be longer than this value.

Step 21 When the DQC experiment has finished, save the data to the hard disk in the Bruker BES3T format (.DSC / . DTA files).

Step 22 Convert the .DSC / .DTA files into ASCII format (.dat) and mirror the DQC trace at the maximum, i.e. average the $+i^{th}$ and $-i^{th}$ data point next to the maximum. Data conversion and mirroring of the time trace can be done automatically in a single step using a Matlab script.[8]

Step 23 Analyse the mirrored DQC trace. In the following, the analysis of DQC data using the DeerAnalysis[9] toolbox for Matlab will be outlined.

I. Import DeerAnalysis into Matlab as described in the user manual of the program. Load the time trace into DeerAnalysis 2022 using ASCII as the input data format.

II. As the modulation depth of the time trace usually amounts to > 90%, the program will issue the message "Data decay to less than 2% of initial amplitude. Background correction switched off", i.e. background correction is by default disabled. Compared to other PDS techniques such as PELDOR or RIDME, the background contribution is considerably smaller in DQC as the phase cycle efficiently suppresses any signal that does not stem from the double quantum coherence pathway.[10] In some cases, however, especially for higher spin concentrations, the intermolecular dipolar coupling becomes significant and the background of the trace cannot be neglected anymore. To still process those traces with DeerAnalysis, changes may be made in the program code to suppress the error message and to allow background correction. For DeerAnalysis 2022, the following procedure proved to be successful: In the file "update_DA.m" within the DeerAnalysis folder, locate the code "if min(td_fit)<0.02". Replace 0.02 by 0.00 and restart DeerAnalysis; this enables background correction.

III. The background in the DQC experiment is not analytically known and depends on the profiles of the MW pulses and the EPR spectrum,[11] i.e. assumptions on the background model have to be made. One option is to fit the background using a homogeneous n-dimensional model or polynomials in such a way that the background-corrected time trace is flat at long dipolar evolution times, i.e. the last quarter of the time trace.[12] The quality of the background removal can be assessed by inspecting the Fourier transform of the time trace (i.e. the Pake pattern).
DeerNet, which transforms the time trace into a distance distribution using neural networks, should only be used with caution as it has not been trained with DQC traces. Thus, errors in the background removal may occur.

IV. Use Tikhonov regularization to translate the dipolar trace into a distance distribution. With DQC traces, DeerAnalysis often yields well-shaped L-curves and choosing a regularization parameter in the corner of the L-curve is usually appropriate. Use the validation routine of DeerAnalysis to inspect the influence of the background correction on the distance distribution. Figure 8 illustrates the procedure of DQC data analysis.

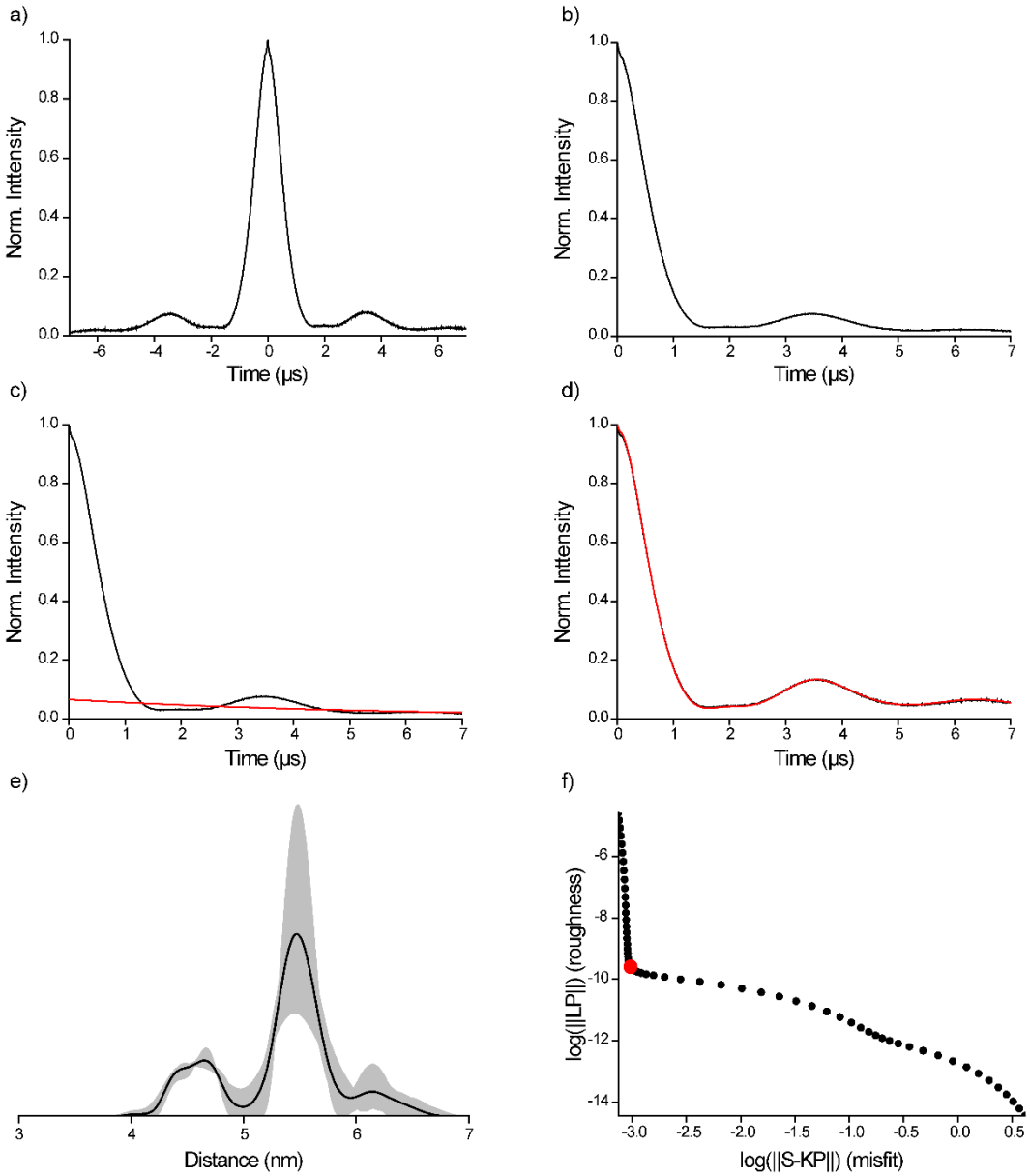

Figure 8: Analysis of DQC time traces. a) Normalized DQC time trace as obtained from the spectrometer. b) DQC time trace from (a), mirrored at the zero-time. c) Mirrored DQC time trace from (b) with a background fit indicated in red. d) Background-corrected time trace obtained by dividing the time trace in (c) by the background fit in (c); the red line indicates a fit to the time trace from Tikhonov regularization. e) Distance distribution (black line) with the uncertainty analysis from the DeerAnalysis validation routine shown as a grey-shaded area. f) L-curve for setting the regularization parameter; the red dot marks the regularization parameter chosen in this example.

Step 24 Interpret the distance distribution using *in silico* spin labelling software like mtsslWizard,[13,14] MMM,[15] or CREST/MD.[16] Translate the results into a structural model of the biomolecule or answer a specific biochemical question in the framework of integrative structural biology.

References

[1] T. Hett, O. Schiemann, PELDOR Measurements on Nitroxide-Labeled Oligonucleotides in *DNAzymes: Methods and Protocols* (Eds. G. Steger, H. Rosenbach, I. Span), *Meth. Mol. Biol*. **2022**, *2439*, Humana New York, pp. 241-274.

[2] M. F. Vicino, T. Hett, O. Schiemann, *Bio-protoc*. **2021**, *11*, e4004.

[3] G. Jeschke, Instrumentation and Experimental Setup in *ESR Spectroscopy in Membrane Biophysics* (Ed. M. A. Hemminga, L. J. Berliner), Springer New York, **2007**, pp. 17-47.

[4] G. Jeschke, Y. Polyhach, *Phys. Chem. Chem. Phys*. **2007**, *9*, 1895-1910.

[5] O. Schiemann, C. A. Heubach, D. Abdullin, K. Ackermann, M. Azarkh, E. G. Bagryanskaya, M. Drescher, B. Endeward, J. H. Freed, L. Galazzo et al., *J. Am. Chem. Soc.* **2021**, *143*, 17875-17890.

[6] H. H. Haeri, P. Spindler, J. Plackmeyer, T. Prisner, *Phys. Chem. Chem. Phys.* **2016**, *18*, 29164-29169.

[7] E. Reijerse, A. Savitsky, *eMagRes* **2017**, *6*, 187-206.

[8] Tobias Hett, "DTA2dat", can be found under https://github.com/TobiasHett/DTA2dat.

[9] G. Jeschke, V. Chechik, P. Ionita, A. Godt, H. Zimmermann, J. Banham, C. R. Timmel, D. Hilger, H. Jung, *Appl. Magn. Reson.* **2006**, *30*, 473-498.

[10] P. P. Borbat, J. H. Freed, *eMagRes* **2017**, *6*, 465-494.

[11] G. Jeschke, *Annu. Rev. Phys. Chem.* **2012**, *63*, 419-446.

[12] N. Fleck, C. A. Heubach, T. Hett, F. R. Haege, P. P. Bawol, H. Baltruschat, O. Schiemann, *Angew. Chem. Int. Ed.* **2020**, *59*, 9767-9772.

[13] G. Hagelueken, D. Abdullin, O. Schiemann, *Methods Enzymol.* **2015**, *563*, 595-622.

[14] G. Hagelueken, R. Ward, J. H. Naismith, O. Schiemann, *Appl. Magn. Reson.* **2012**, *42*, 377-391.

[15] Y. Polyhach, E. Bordignon, G. Jeschke, *Phys. Chem. Chem. Phys.* **2011**, *13*, 2356-2366.

[16] S. Spicher, D. Abdullin, S. Grimme, O. Schiemann, *Phys. Chem. Chem. Phys.* **2020**, *22*, 24282-24290.