Journal of Visualized Experiments

Paramagnetic Relaxation Enhancement for Detecting and Characterizing Self-Associations of Intrinsically Disordered Proteins.

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63057R2
Full Title:	Paramagnetic Relaxation Enhancement for Detecting and Characterizing Self-Associations of Intrinsically Disordered Proteins.
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biochemistry
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	San Antonio, TX, USA
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TITLE:

Paramagnetic Relaxation Enhancement for Detecting and Characterizing Self-Associations of Intrinsically Disordered Proteins.

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SUMMARY:

A protocol for the application of paramagnetic relaxation enhancement NMR spectroscopy to detect weak and transient inter- and intra-molecular interactions in intrinsically disordered proteins is presented.

ABSTRACT:

Intrinsically disordered proteins and intrinsically disordered regions within proteins make up a large and functionally significant part of the human proteome. The highly flexible nature of these sequences allows them to form weak, long-range, and transient interactions with diverse biomolecular partners. Specific yet low-affinity interactions promote promiscuous binding and enable a single intrinsically disordered segment to interact with a multitude of target sites. Because of the transient nature of these interactions, they can be difficult to characterize by structural biology methods that rely on proteins to form a single, predominant conformation. Paramagnetic relaxation enhancement NMR is a useful tool for identifying and defining the structural underpinning of weak and transient interactions. A detailed protocol for using paramagnetic relaxation enhancement to characterize the lowly-populated encounter complexes that form between intrinsically disordered proteins and their protein, nucleic acid, or other biomolecular partners is described.

INTRODUCTION:

Intrinsic disorder (ID) describes proteins (IDPs) or regions within proteins (IDRs) that do not spontaneously fold into stable secondary or tertiary structures but are biologically active. Generally, the function of IDP/IDRs is to facilitate specific yet reversible interactions with biomolecules at physiological conditions[1]. Thus, IDPs and IDRs are involved in a range of cellular functions, including recruitment, organization, and stabilization of multi-protein complexes, for example, the assembly and activity of the spliceosome[2], recruitment and organization of

components at sites of DNA damage[3], organization, and stabilization of the recruitment of transcription complexes[4], or of the chromatin remodeler BAF[5]. Additionally, IDPs are found at signaling nexuses where their promiscuity for different binding partners enables them to mediate information transfer through cellular protein networks[6]. Recent work has also revealed a proclivity for IDR regions to self-associate forming biomolecular condensates through the process of liquid-liquid phase separation[7]. Many of the aforementioned functions involving ID are also now thought to involve some aspect of condensate formation[8]. Despite the importance of ID for biomolecular complex assembly, stabilization, scaffolding, and signal transduction, the atomic details of their specific interactions are difficult to identify since IDPs and IDRs are typically not amenable to structural investigations using x-ray crystallography and cryogenic electron microscopy.

Nuclear magnetic resonance (NMR) is an ideal technique for investigating ID as it is not dependent on the presence of rigid or homogenous structural ensembles but reports on the immediate local environment of individual nuclei. The resonant frequency, or chemical shift, of a nucleus in a given molecule is influenced by weak magnetic fields induced by the local electronic distribution, which in turn is dependent on bond lengths, angles, nearness of other nuclei, interactions with binding partners, and other factors[9]. Thus, each nucleus acts as a unique, site-specific structural probe sensitive to changes in its local chemical environment. Despite these advantages, NMR is a bulk technique, and the observed chemical shift is the average of all the environments sampled by a particular nucleus. A range of NMR techniques, many of which are described in this issue, have been developed to recover structural, dynamic, and kinetic information about high energy, lowly-populated biomolecular conformations contained in the averaged chemical shift[10,11]. Although transiently populated, identification and quantification of these states are important for determining the details of functional mechanisms[12]. For example, in the case of IDPs and IDRs, the conformational ensemble may be biased to preferentially sample conformations that are productive for the formation of encounter complexes with physiological binding partners. Detection of these states, as well as identification of the residue-specific inter- and intra-molecular interactions and dynamics, are important to determine the underlying structural mechanisms of protein function and complex formation.

A protocol for using paramagnetic relaxation enhancement (PRE) NMR to investigate transient, lowly-populated states important for the formation of IDP/IDR-mediated biomolecular complexes is described[13]. This approach is useful for studying the transient protein-protein interactions such as those that promote the assembly of amyloid fibrils from ID a-synuclein[14,15] or the self-association of FUS[16], as well as to characterize specific protein-protein interactions such as between signaling proteins[17]. An example of a self-associating IDP is presented, where specific inter- and intra-molecular interactions result in preferentially compacted states as well as site-specific interactions that drive self-association. The PRE arises from the magnetic dipolar interaction of a nucleus to a paramagnetic center with an isotropic *g*-tensor, commonly supplied in the form of an unpaired electron on a nitroxide group or as a paramagnetic metal atom[18] (**Figure 1**). While atoms with anisotropic *g*-tensors also produce a PRE effect, analysis of these systems is more difficult due to confounding effects contributed by the pseudo-contact shifts (PCS) or residual dipolar coupling (RDC)[13,19]. The strength of the interaction between a nucleus

and the paramagnetic center is dependent on the $\langle r^{-6} \rangle$ distance between the two. This interaction results in an increase in nuclear relaxation rates, which causes detectable line broadening even for long-range interactions (~10–35 Å), because the magnetic moment of the unpaired electron is so strong[20,21]. Detection of transient states with the PRE is possible if the following two conditions are met; (1) the transient interaction is in fast exchange on the NMR timescale (observed chemical shift is a population-weighted average of the exchanging states); and (2) the nuclei to paramagnetic center distance is shorter in the transiently populated state than in the major state[11]. The transverse PRE is denoted Γ_2 and, for practical purposes, is calculated from the difference in ^{1}H transverse relaxation rates between a sample containing a paramagnetic center and a diamagnetic control. For an in-depth treatment of the theory of the PRE and related pseudocontact shifts in fast and slow exchange regimes, the reader is referred to the comprehensive reviews by Clore and co-workers[13,22]. Here, only the situation where ^1H_N-Γ_2 is in the fast exchange regime is considered, where because of the r^{-6}-dependence of the PRE, the observed relaxation rate is related to *both* the distance to which the paramagnetic center approaches the nucleus as well as the amount of time it spends in that conformation. Therefore, transient conformations that do not involve a close approach produce a small PRE while closer interactions, even if short-lived, will produce a larger PRE.

For IDPs, the PRE is used to measure and differentiate the interactions occurring within a single molecule (intramolecular) and between separate molecules (intermolecular). By attaching a paramagnetic center to an NMR visible (e.g., ^{15}N-labeled) or NMR invisible (e.g., natural abundance ^{14}N) protein, the source (inter- or intra-molecular) of the PRE may be determined (**Figure 2**). Site-directed mutagenesis that introduces a cysteine residue is a convenient approach to attach a paramagnetic center (spin-label) to a protein[23]. Several types of molecules have been proposed for use as spin labels, including metal chelating (EDTA-based) and free-radical (nitroxide-based)[24]. Various nitroxide spin labels have been described and are available with different cysteine-reactive chemistries such as methanethiosulphonate, maleimide, and iodoacetamide[25,26] (**Figure 1**). Inherent flexibility of the tag or of the linker may be problematic for certain analyses, and in these situations, different strategies have been proposed to limit the motion of the tag, such as by adding bulky chemical groups or the use of a second linker to anchor the tag to the protein (two site attachment)[27,28]. Additionally, commercially available tags may contain diastereomeric proteins but generally this will not contribute to the observed PRE[29]. The use of the 3-Maleimido-PROXYL attached to a free cysteine via maleimide chemistry is described since it is readily available, cost-effective, non-reversible, and the reducing agent tris(2-carboxyethyl)phosphine (TCEP) can be maintained in the solution throughout the labeling reaction. Since 3-Maleimido-PROXYL has an isotropic *g*-tensor, no PCS or RDCs are induced, and the same chemical shift assignments can be used for both the paramagnetic and diamagnetic samples[13].

The ^1H_N-T_2 is measured using a two time-point strategy (T_a, T_b) that has previously been shown to be as accurate as collecting a full evolution series consisting of 8 to 12 time points[30]. The first time point (T_a) is set as close to zero as practical, and the optimal length of the second time point is dependent on the magnitude of the largest expected PRE for a given sample and can be estimated from: $T_b \sim 1.15/(R_{2,dia} + \Gamma_2)$ where $R_{2,dia}$ represents the R_2 of the diamagnetic sample[13].

If the magnitude of the largest PREs is unknown, setting T_b to ~ one times the ^{1}H T_2 of the protein is a good initial estimate and further optimized by adjusting T_2 to improve the signal to noise. This two-point measurement strategy significantly reduces the experimental time required to measure PREs and allows time for more signal averaging, particularly since relatively dilute samples are used to minimize the effects of non-specific contacts between molecules. An HSQC-based pulse sequence is used to measure ^1H_N-T_2 and has been described in detail elsewhere[30]. For improved sensitivity, the hard pulses of the forward and back INEPT transfers may be replaced with shaped pulses; alternatively, the sequence is readily converted to a TROSY-based readout[31]. Since IDPs typically have much longer transverse relaxation rates resulting in narrower line widths (due to the inherent disorder) than similarly sized globular proteins, long acquisition times in the indirect dimension may be used to improve spectral resolution and alleviate the chemical shift dispersion limitation inherent for IDPs.

PRE is a useful tool for studying protein-protein and protein-nucleic acid interactions, particularly interactions that are transient or lowly populated. A detailed protocol for the preparation of an NMR sample suitable for measuring PREs, including steps for protein purification, site-directed spin labeling, setting up and calibrating the pulse program, processing, and interpreting the NMR data, is provided. Important experimental considerations are noted throughout that may impact data quality and experimental outcome, including sample concentration, selection of the spin-label, and removal of paramagnetic components.

PROTOCOL:

General requirements for the protocol: protein purification facilities, UV-Vis spectrometer, high-field NMR spectrometer and operating software, post-processing analysis software including; NMRPipe[32], Sparky[33], (or CCPN Analysis[34], or NMRViewJ[35]).

1. Recombinant expression and purification of a protein for PRE measurements

1.1. Design an expression construct for the protein of interest so that there is a single cysteine residue present. Multiple mutations will be required to introduce a free cysteine at different positions in the protein of interest[36].

1.2. Express and purify a natural abundance (^{14}N) or ^{15}N-labeled sample of the protein of interest using an established protocol[37].

NOTE: *E. coli* expression systems provide cost effective and robust for recombinant protein expression since isotopic enrichment of ^{15}N is a minimal requirement for biomolecular heteronuclear NMR spectroscopy. Typical steps are expression in minimal media, chromatographic purification, and removal of affinity purification tag. This protocol assumes a robust expression and purification protocol has been established that can produce sufficient protein of suitable quality for NMR investigations.

1.2.1. Maintain 1 mM reducing agent (DTT or TCEP) in buffers at all purification steps to prevent reaction of the free cysteine and formation of intermolecular disulfide bonds for IDPs.

NOTE: Some systems may be more tolerant and less aggregation prone to non-reducing conditions depending on the specific characteristics of the protein, as well as the temperature, pH, and buffer system chosen for purification[38].

1.2.2. Remove affinity tags used for purification before proceeding since they may non-specifically interact with the protein in unpredictable ways or possibly contain reactive cysteine residues that could inadvertently serve as an unintended attachment site.

1.2.3. Prepare a ^{15}N labeled reference sample without cysteine mutation(s) sample mixed with a soluble version of the spin-label to assess the contribution of solvent PREs.

2. Conjugating the 3-Maleimido-PROXYL nitroxide spin label

2.1. Store or exchange the purified protein into a degassed buffer containing 50 mM Tris pH 7 and 1 mM TCEP; the buffer may also contain up to 8 M urea if needed to aid protein solubility. Alternatively, rapidly dilute a protein stock solution into at least 10 volume equivalents of degassed 50 mM Tris pH 7 and 1 mM TCEP buffer. Ensure that the protein concentration prior to adding spin-label is at least 100 µM.

2.2. Add 3-Maleimido proxyl from a stock solution to 20x molar excess of the protein of interest. Protect the sample from light and oxygen and incubate overnight at room temperature or 4 °C; gentle rocking or nutation may improve labeling efficiency.

2.3. Prepare the stock solutions of the spin-label by dissolving 3-Maleimido proxyl powder in 95% ethanol. Aliquots of the stock can be stored at -80 °C for less than 6 months.

2.4. Critical step: Remove the non-reacted free spin-label to prevent non-specific solvent PREs. Achieve this by gel filtration or (preferably) extensive dialysis of the protein sample. This step will also introduce the protein into a buffer suitable for NMR.

NOTE: Reducing agents should be prepared fresh, and compatibility between buffer components should be considered; for example, TCEP degrades quickly in phosphate-based buffers, and this combination should be avoided[39].

2.5. Treat all buffers used from this step forward with a chelating resin selective for divalent and transition metals to remove paramagnetic ions or spin-label quenchers. If the protein cannot be stored in an NMR buffer, concentrate the protein to be rapidly diluted into a buffer suitable for NMR.

2.6. Monitoring the efficiency of spin-label incorporation.

2.6.1. Use Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid) for quantifying free sulfhydryl groups in solution[40].

NOTE: Detailed protocols are available from the manufacturer. For the purposes here, it is important to determine incorporation of the spin-label, the concentration of free sulfhydryl groups is compared with the total protein concentration. The percent of free sulfhydryl groups is the percent of molecules that do not have a nitroxide spin label attached.

2.6.2. Monitor the intensity of the peak corresponding to the tagged cysteine residue to judge spin-label incorporation into the protein of interest.

NOTE: This is a rapid and effective approach to determine the degree of spin labeling of the protein. Complete incorporation of the spin-label will result in the disappearance of the peak from the spectrum. With the poor dispersion characteristic of IDPs the peak corresponding to the mutant cysteine residue may not always be readily identified, and thus the use of Ellman's reagent (step 2.6.1) is recommended.

3. Prepare NMR sample for measuring intra- or inter-molecular PRE

3.1. Prepare sample for measurement of intramolecular PRE

3.1.1. Prepare ^{15}N isotopically enriched, spin-labeled protein to a concentration of at least 100 µM but not more than 300 µM in a buffer suitable for NMR. Total sample volume (including D_2O) is 500 – 550 µL.

NOTE: Common NMR buffers include phosphate, acetate, (bi)carbonate, and TRIS. Good's buffers such as MES, HEPES may also be appropriate. Exercise caution when selecting buffers to ensure no cross-reactivity with other solution components.

3.1.2. Ensure that the pH is ~7.2 or lower to minimize the effects of amide proton exchange with water. Keep the concentration of salt as low as possible (typically less than 150 mM), although the primary consideration is to maintain protein stability.

NOTE: Approaches for conducting NMR experiments in high-salt conditions have been described elsewhere[41].

3.2. Prepare sample for measurement of intermolecular PRE

3.2.1. Follow this step or step 3.1; they are not performed simultaneously. Prepare ^{14}N natural abundance, spin-labeled protein in the chosen NMR buffer.

3.2.2. Prepare the protein sample by mixing ^{15}N isotopically enriched non-spin-labeled protein with 1%–50% ^{14}N natural abundance spin-labeled protein so that the final concentration is

identical to the sample prepared in 3.1.1. The total sample volume (including D_2O) is 500 – 550 µL.

3.2.3. Empirically optimize the ratio of the ^{15}N and ^{14}N proteins for each protein studied. The ratios of 1%, 5%, and 20% of ^{14}N-spin-labeled protein are good starting points.

NOTE: A buildup of the PRE as a function of added ^{14}N-spin-labeled protein indicates a specific effect; the observed PRE is sample-specific since it depends on distance and population (as discussed above), and therefore higher ratios of ^{14}N-spin-labeled protein will be required if the interaction is particularly transient[17].

3.3. Transfer the NMR (either intra- or inter-molecular) sample to a 5 mm NMR tube that is appropriate for use in high-field magnets using a long-stem (9”) glass pipette or micropipette. Ensure that all NMR samples include 5%–10% of D_2O to facilitate field locking.

NOTE: NMR tubes that utilize polymer plugs to reduce the necessary sample volume are not recommended for PRE measurements due to difficulties related to effective sample shimming.

4. Set up NMR spectrometer and experiment specific parameters

4.1. Exercise extreme caution when working around superconducting, high-field NMR spectrometers.

NOTE: Hazards include injuries due to the sudden acceleration of metallic objects toward the magnet, interference with implanted medical devices, and asphyxiation due to a sudden release of N_2 and He_2 gas in the event of a magnet quench. The following steps assume that the reader has undergone the required training, is aware of these and other local hazards, and has received approval from the facility manager to operate the NMR spectrometer. When in doubt of a step or instruction, consult with the facility manager or experienced user to prevent potential personal injury or damage to the spectrometer.

4.2. The following steps assume a commercial NMR spectrometer running a modern version of the acquisition control software. Download the pulse program and parameter files and place them in the appropriate directories.

NOTE: A pulse program and parameter set suitable for use with a Bruker spectrometer and TopSpin (3.2 or later) are available upon request from the authors.

4.2.1. Critical step: Familiarity with installing non-native NMR pulse programs is assumed; consult with the facility manager or an experienced user if necessary.

4.3. Place the sample in the magnet, lock on the ^{2}H signal using the ***Lock*** command, tune and match the ^{1}H channel according to facility protocols (the exact procedure will depend on if the probe is equipped with a remote tune and match module).

4.4. Adjust the shims using the ***topshim*** subroutine to optimize solvent signal suppression.

4.5. Calibrate the ^{1}H and ^{15}N 90° pulses using standard methods.

4.5.1. Calibrate the ^{1}H pulse using the ***popt*** program (use ***pulsecal*** first to estimate pulse length).

4.5.2. Calibrate the ^{15}N pulse against a standard sample; make sure this value has been calibrated recently by discussing with a technical director or experienced user.

4.5.3. Alternatively, calibrate the ^{15}N pulse on the sample by varying one of the 90° pulses of an HMQC experiment until a null signal is achieved.

4.5.4. Determine the correct attenuation for shaped pulses using the shape tool (***stdisp***) subroutine.

4.5.5. Open the appropriate pulse shape file by clicking on the folder icon. The shaped pulses are found in the pulse parameters section of **ACQUPARS.**

4.5.6. Load the pulse definition file and click on **Analyze Waveform** > **Integrate Shape**. Input the calibrated ^{1}H 90° hard pulse, desired shaped pulse length, and rotation (90° or 180°).

4.5.7. Calculate the power level of the shaped pulse by adding the change of power level to the attenuation for the calibrated 90° pulse.

4.6. Record a standard ^{1}H, ^{15}N HSQC (hsqcetfpf3gpsi) to optimize sweep width, carrier frequency and check water suppression[25].

4.7. Adjust the sweep width and the number of indirect dimension increments using the ***sw*** and ***td*** commands or directly in the appropriate dialog boxes. Typically, for collecting PREs, spectral widths are chosen so that the spectrum is not folded.

5. Setup the $^{1}H_N$-T_2 experiment

5.1. Calibrate the shaped pulses as described above (4.4.5–4.5.7). The shaped pulse parameter files for the PRE experiment are Eburp2.1000 (90° pulse), Reburp.1000, and Iburp2.1000. Enter the calibrated pulse lengths in the pulse parameters section on the **ACQUPARS** tab.

5.2. This experiment measures the $^{1}H_N$-T_2 using the two time-delay point approach[30].

5.2.1. Set the time delays by editing the ***vdlist*** file, the first delay (T_a) is set to 0.01 ms.

5.2.2. Choose the second delay, (T_b) using the relationship to the expected maximum PRE ($T_b \sim 1.15/(R_{2,dia} + \Gamma_2)$ where $R_{2,dia}$ represents the R_2 of the diamagnetic sample[13]. Without prior

knowledge of the magnitude of the PRE contribution to the observed relaxation, a good starting point is setting T_b to ~1x ^{1}H T_2.

5.2.3. Then determine a suitable value by comparing the first increments (processed with the ***efp*** command) of the T_a and T_b spectra and adjusting T_b such that the signal decays to between 40%–50% of its initial value.

NOTE: This approach optimizes the spectral signal-to-noise, a necessary consideration for samples that cannot be highly concentrated (< 50 µM). Suitable values of T_b are sample dependent but typically range from 8 - 40 ms for an average sized protein.

5.3. Determine the number of complex points to record and number of scans for sufficient signal averaging. Since IDPs have longer ^{15}N T_2 than folded proteins of comparable size, longer acquisition times in the indirect dimension may be used.

NOTE: This value is dependent on the specific characteristics of the protein but can be roughly estimated from the ^{15}N T_2 and optimized by monitoring signal decay in the FID. For the direct dimension, 1024* complex points (13 ppm sweep width, 112.6 ms acquisition time) is sufficient for most samples.

5.4. Use the command ***expt*** to calculate the experiment time and then start the experiment with the command *zg.*

6. Make a diamagnetic sample by reducing spin-label with ascorbic acid

6.1. Dissolve sodium ascorbate in the NMR buffer and adjust the pH to match the original NMR buffer.

6.2. Calculate the concentration of sodium ascorbate stock so that a 10x molar excess of ascorbate over the concentration of the spin-label can be added with the least change of sample volume. For example, for a 100 µM protein sample, a 100 mM stock of ascorbate is appropriate. Reducing the spin-label will require adding 5.5 µL of ascorbic acid stock solution, which is only 1 % of the total sample volume.

6.3. Add the required amount of ascorbic acid to the NMR tube by placing a droplet below the rim of the tube, cap the tube, carefully invert the tube to mix, and then spin at 200–400 x *g* for 10–20 s in a hand-cranked centrifuge to settle the sample at the bottom of the tube.

6.4. Wrap the NMR tube in foil to protect from light and allow the reaction to proceed for at least 3 h.

6.5. Record ^1H_N-T_2 on the diamagnetic sample using the same parameters used for the paramagnetic sample.

6.6. Recalibrate the pulses. However, they should not have changed from the paramagnetic measurements; if they are significantly different (> 0.5 µs difference), consider sample quality (e.g., degradation, precipitation).

6.7. Ensure that all acquisition parameters, including the specified relaxation delays (***vdlist***), number of dummy scans, number of scans collected, number of complex points collected, acquisition time, sweep widths, and carrier frequencies remain the same for the diamagnetic and paramagnetic samples.

7. Process paramagnetic and diamagnetic spectra

7.1. Copy the data to local computer or workstation that has NMRPipe and Sparky installed and configured. Make a folder named ***proc*** in the experiment data directory that contains the ***ser*** file.

7.2. Copy the NMRPipe scripts ***fid.com***, ***p3d.com***, and ***nmrproc.com*** into ***proc*** (processing scripts are available upon request from the authors).

7.2.1. Use the ***fid.com*** script to convert the Bruker data format (***ser***) into NMRPipe format.

7.2.2. Use the ***p3D.com*** script to split the pseudo3D planes into individual spectra.

7.2.3. Use the ***nmrproc.com*** script to read the output of the ***fid.com*** script, apply solvent suppression, a window function, append zeros to the raw data (zero fills), apply phase correction, execute a Fourier transformation, trim the data for display and write the processed data to disk. The script will output one file for each relaxation delay recorded (T_a and T_b).

NOTE: Each of these scripts is customizable to optimize the processing for the specific details of each experiment. Tutorials and example data sets are included in the NMRPipe distribution available from the NMRPipe website[32]. NMRDraw may be used for spectral viewing during processing (e.g., setting proper phase angles etc.). Options available for NMRPipe commands can be viewed using the command ***nmrPipe -help***.

8. Transfer resonance assignments and extract peak heights

8.1. Change the file header information for each spectrum file (T_a, T_b for both paramagnetic and diamagnetic samples) using the command ***sethdr* [filename] *-ndim 2***.

8.2. Use Sparky to extract peak heights[33] following steps 8.3–8.5. Other software packages, including NMRPipe (NMRDraw)[32], CCPN Analysis[34], and NMRViewJ[35] are also appropriate.

8.3. Read the spectral files into Sparky. At this step the data set will consist of one spectrum for each time point spectra (T_a, T_b), for both the paramagnetic and diamagnetic samples, measured for each position of the spin-label in the protein.

8.4. Use Sparky to pick peaks (command: ***F8,*** then ***click and drag***) and transfer assignments using the ***transfer peak list tool*** from a reference peaklist.

NOTE: Resonance assignments of the protein of interest are necessary for sequence-specific interpretation of the observed PREs[36].

8.4.1. Set contours in both paramagnetic and diamagnetic spectra to the same level. Ensure to set the contours so that the spectra collected after the time delay do not purposely exclude peaks but are high enough so that the T_b spectra are not overly noisy.

8.5. Save the new peak lists for each spectrum and include the measured peak intensity and Sparky calculated signal to noise ratio (command: ***it*** to open peaklist, click options to include ***intensity*** and ***SNR*** columns, command: ***save***).

9. Extract ^1H_N-T_2 rates for each residue and calculate PRE

9.1. Import the peak lists into spreadsheet software or a preferred programming language such as Python.

NOTE: For each spin-label position on the protein, the dataset will consist of four peak lists with associated peak intensities, one each of T_a and T_b for both the paramagnetic and diamagnetic experiments.

9.2. Calculate ^1H_N R_2 for both the paramagnetic and diamagnetic samples using the equation:

$$R_2 = \frac{1}{\Delta T} \cdot \ln \frac{\text{peak intensity } (T_a)}{\text{peak intensity } (T_b)}$$

$$\text{where } \Delta T = T_b - T_a$$

9.3. Use the above equation to determine the relaxation rate for each residue for the paramagnetic and diamagnetic samples.

9.4. Determine the ^1H_N-Γ_2 rate for each residue using the equation:

$$\Gamma_2 = R_{2(paramagnetic)} - R_{2(diamagnetic)}$$

9.5. Use the Sparky calculated signal to noise ratio (SNR) to compute the uncertainty of the peak height for each residue.

9.6. Propagate the error using the equation:

$$\sigma = \frac{1}{\Delta T} \cdot \sqrt{\left\{\frac{\sigma_{dia}}{I_{dia}(T_a)}\right\}^2 + \left\{\frac{\sigma_{dia}}{I_{dia}(T_b)}\right\}^2 + \left\{\frac{\sigma_{para}}{I_{para}(T_a)}\right\}^2 + \left\{\frac{\sigma_{para}}{I_{para}(T_b)}\right\}^2}$$

9.7. Plot ^1H_N-Γ_2 as a function of residue number using a scatter plot including the error calculated in 9.6.

REPRESENTATIVE RESULTS:

Intramolecular $^{1}H_N$-Γ_2 PREs were recorded on a self-associating, intrinsically disordered fragment (residues 171-264) derived from the low-complexity domain of the RNA-binding protein EWSR1[42] (**Figure 3**). Residues in close sequential proximity to the spin-label attachment point (e.g., residue 178 or 260 in **Figure 3**) are expected to be significantly broadened and are not detectable in the spectrum. Residues sequentially spaced from the attachment point yet show enhanced Γ_2 were spatially close (10–35 Å) to the spin-label. In the case of EWSR1 171-264, attributing the source of the PRE effect is complicated since it may arise from a combination of inter- and intra- residue contacts and is dependent on the distance from the nucleus to the paramagnetic center, the population of that conformation, and the dynamics of the vector connecting the electron and nuclear spins. Further, the magnitude of PREs arising from intramolecular contacts is not concentration-dependent, while PREs arising from intermolecular contacts depend on concentration as well as the kinetics and dynamics of the association between protein molecules.

A possible interpretation of these data is that the IDP ensemble samples conformations that are more compact than an extended chain. Alternatively, the PREs could arise from intermolecular contacts responsible for the self-association of EWSR1, or the PREs could be from a combination of both intra- and intermolecular contacts. In the case presented here, what remains unknown is how close the residues approach the spin-label or for how long they remain in close proximity. With highly flexible molecules such as EWSR1 171-264, it can be difficult to qualitatively disentangle these parameters. By placing the spin-label at different residue positions, contacts between different parts of the chain may be identified, providing a more accurate interpretation of specific interactions that may be functionally relevant for self-association (**Figure 3**). Measuring intermolecular PREs (^{14}N spin-labeled protein mixed with ^{15}N non-spin labeled protein), employing a mutational strategy of residues with larger than average PREs (e.g., residues 196 or 215, **Figure 3**), and utilizing other biophysical methods such as dynamic light scattering, size exclusion chromatography, and analytical ultracentrifugation, are useful for characterizing the conformational ensemble of an IDP.

FIGURE AND TABLE LEGENDS:

Figure 1: Molecules containing an unpaired electron and various functional groups to facilitate attachment to free cysteine residues that are typically used as paramagnetic relaxation agents. Diamagnetic molecules may be used as controls. (**A**) 3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, free radical (3-Maleimido-PROXYL) (**B**) 3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, free radical (3-Carboxy-PROXYL) (**C**) 3-(2-Iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, free radical (3-(2 – Iodoacetamido-PROXYL) (**D**) 1-Oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl) Methyl Methanethiosulfonate (MTSL) (**E**) (1-Acetoxy-2,2,5,5-tetramethyl-δ-3-pyrroline-3-methyl) Methanethiosulfonate (Acetoxy-MTSL)

Figure 2: Depiction of intra- and intermolecular PRE. (**A**) Intramolecular PRE, the red circle represents the effective radius of a paramagnetic center attached to a ^{15}N-labeled protein. The PRE effect decreases with an $\langle r^{-6} \rangle$ dependence on distance from the paramagnetic molecule. (**B**) Intermolecular PRE, the paramagnetic group (red circle), is located on a ^{14}N (natural abundance)

protein (blue) that is invisible to NMR. The effects of the paramagnetic group on the non-NMR active protein are observed as increased relaxation rates when it comes into close contact with the ^{15}N protein (black).

Figure 3: $^{1}H_N$-Γ_2 rates for residues 171-264 of the intrinsically disordered domain of EWSR1. A serine residue at position (**A**) 178 or (**B**) 260 that has been mutated to a cysteine serves as the attachment point for a 3-Maleimido-PROXYL spin-label (red *). Increased relaxation rates occur at the location of the tag, other sites of increased relaxation are indicative of intramolecular interactions.

DISCUSSION:

A method for characterizing transient interactions that exist at low populations between intrinsically disordered proteins and various binding partners using PRE has been presented. In the example shown, the protein is self-associating, and thus the PRE may arise from a combination of inter and intramolecular interactions. This method is readily extended to heterogeneous samples where the interactions between two different proteins may be characterized. Complementary information about how different regions of the protein interact is available by placing the spin-label at different positions within the protein. Additionally, by alternating the spin-label between NMR active (^{15}N) and NMR inactive (^{14}N) species, the intra- and inter-molecular sources of observed PRE may be differentiated from one another, providing information about encounter complexes. The experiment outlined here can report on encounter complex interactions even if they occur on a microsecond timescale[13].

Central to this method is the incorporation of a spin-label tag into the protein of interest by attachment to a cysteine residue. Some proteins may contain a native cysteine that is suitable (does not participate in disulfide bonds, is surface exposed) for attaching a spin-label. For IDPs, solvent exposure of cysteine is usually not an issue. In the majority of cases, it is desirable to introduce cysteines as conservative mutations (serine to cysteine or other uncharged polar amino acids to cysteine) using site-directed mutagenesis[43]. In the example presented, the fragment of EWSR1 does not contain native cysteines and is enriched in serines; thus, devising a mutational strategy was straightforward. Proteins that contain native cysteine(s) present a more complicated case, and care needs to be taken to not disrupt the native function (e.g., break a structurally important disulfide bond)[44]. Further, to incorporate a single cysteine for spin-labeling, the native cysteines must be mutated to a residue that does not react with the spin-label (no mercapto group) and based on its size and other properties, serine is a good substitute for cysteine. If native cysteines need to be mutated, careful characterization of the mutants is required to ensure they maintain native structure and function is essential. Simple ^{1}H,^{15}N HSQCs of mutants in comparison to the wildtype protein are powerful indicators of perturbations (even minor) to protein structure, and this approach is also useful for IDPs[45]. Other methods to consider are circular dichroism, analytical ultracentrifugation, or biochemical approaches such as activity assays[46].

Technical considerations for obtaining reproducible, rigorous, high-quality data include the removal of ionic impurities during the preparation of the NMR sample. This is achieved by passing

all solutions over chelating resin prior to use. Further, using a properly degassed buffer is important during the attachment of the nitroxide spin label as the presence of oxygen can reduce the efficiency of labeling. Diamagnetic contamination will contribute to a decrease in the observed PRE; however, the effect is less pronounced on intramolecular PREs and can be reduced by decreasing ΔT[13]. Therefore, it is not necessary to obtain 100 % label incorporation to proceed with the experiment, particularly for the qualitative interpretation presented here. If free cysteines from incomplete spin-label attachment are problematic, some mercapto-reactive chemistries (e.g., maleimide) are amenable to maintaining a reducing agent in the sample throughout the experiment[26]. It is important that the paramagnetic and diamagnetic samples match each other as closely as possible. When reducing the spin-label with ascorbic acid to create a diamagnetic control, consider the dilution factor introduced from titrating in an ascorbic acid stock solution. This dilution can be minimized by maintaining the ascorbic acid stock at least 10x the expected working concentration in the NMR buffer.

There are many software packages available for analyzing NMR data, including NMRPipe[32], Sparky[33], CCPN Analysis[34], NMRViewJ[35], among others. The combination of NMRPipe for spectral processing and Sparky for spectral analysis (peak picking and quantification) was described here due to the ease of use of this combination. NMRPipe is commonly used by many NMR groups for spectral processing, but the NMRPipe suite contains the tools necessary for completing all steps of the analysis, albeit with a significant learning curve. Data may also be processed using the NMR spectrometer control software. Sparky was chosen for spectral analysis because of its ease of use and rapid uptake by novice users. There are several options available for spectral analysis (peak picking and measuring peak heights) that can easily substitute for the functionality of Sparky including CCPN Analysis, or NMRViewJ. Notably, many of these programs have overlapping functionalities and the user is advised to select the combination of programs with which they are most comfortable.

Poor chemical shift dispersion is an inherent problem with IDPs leading to significant resonance overlap and introduction of error into the measurement of peak height. Different strategies have been proposed to alleviate this problem. One of the most straightforward, and the one employed here, is to take advantage of the long transverse relaxation characteristic of IDPs and simply extend the acquisition time in the ^{15}N (indirect) dimension. Alternatively, the triple resonance HNCO experiment is useful for resolving resonance overlap in IDPs due to the superior dispersion of C' resonances. Both TROSY and HSQC versions of the HNCO for measuring PREs have been proposed and are described elsewhere[47]. However, the improved resolution is not always significant enough to warrant the increased complexity of the experiment, longer time for data collection, and added cost for preparing a suitable sample (enrichment of ^{13}C). This is indeed the case for EWSR1 171-264 presented here, where no significant improvement in the number of non-overlapped residues was observed between a TROSY-HNCO and an ^{1}H, ^{15}N HSQC collected with long acquisition time in the indirect dimension.

This procedure outlined above focuses on the utility of PRE experiments for characterizing weak interactions that exist within and between intrinsically disordered proteins. The PRE has a much broader utility in biomolecular NMR, including determining long-range structural restraints and

quantitative determination of sparsely populated conformational states. For example, Clore and co-workers have pioneered the use of the PRE to detect and quantify transient interactions arising from interactions between individual domains of a single protein[48] or between the subunits of assembled protein complexes[17]. There are many examples of the PRE used to derive long-range distance restraints, including for large proteins[49], or with novel PRE tags[50], to help determine the overall fold of a protein[51], as well as in highly paramagnetic systems[52]. Finally, while PCS is beyond the scope of this discussion, they have been applied to important biomolecular problems which have been described elsewhere[53]. The method presented above is suitable for probing the conformation and interactions of IDPs using PREs and was designed to be accessible for novice users. For more quantitative approaches to the analysis of the PRE, the user is referred to the many excellent articles referenced within[11,24,30,31].

ACKNOWLEDGMENTS:

We thank Drs. Jinfa Ying and Kristin Cano for helpful discussions and technical assistance. DSL is a St. Baldrick's Scholar and acknowledges the support of the St. Baldrick's Foundation (634706). This work was supported in part by the Welch Foundation (AQ-2001-20190330) to DSL, the Max and Minnie Tomerlin Voelcker Fund (Voelcker Foundation Young Investigator Grant to DSL), UTHSA Start-Up Funds to DSL, and a Greehey Graduate Fellowship in Children's Health to CNJ. This work is based upon research conducted in the Structural Biology Core Facilities, part of the Institutional Research Cores at the University of Texas Health Science Center at San Antonio supported by the Office of the Vice President for Research and the Mays Cancer Center Drug Discovery and Structural Biology Shared Resource (NIH P30 CA054174).

DISCLOSURES:

All authors have read and approved the manuscript. No conflicts of interest are declared.

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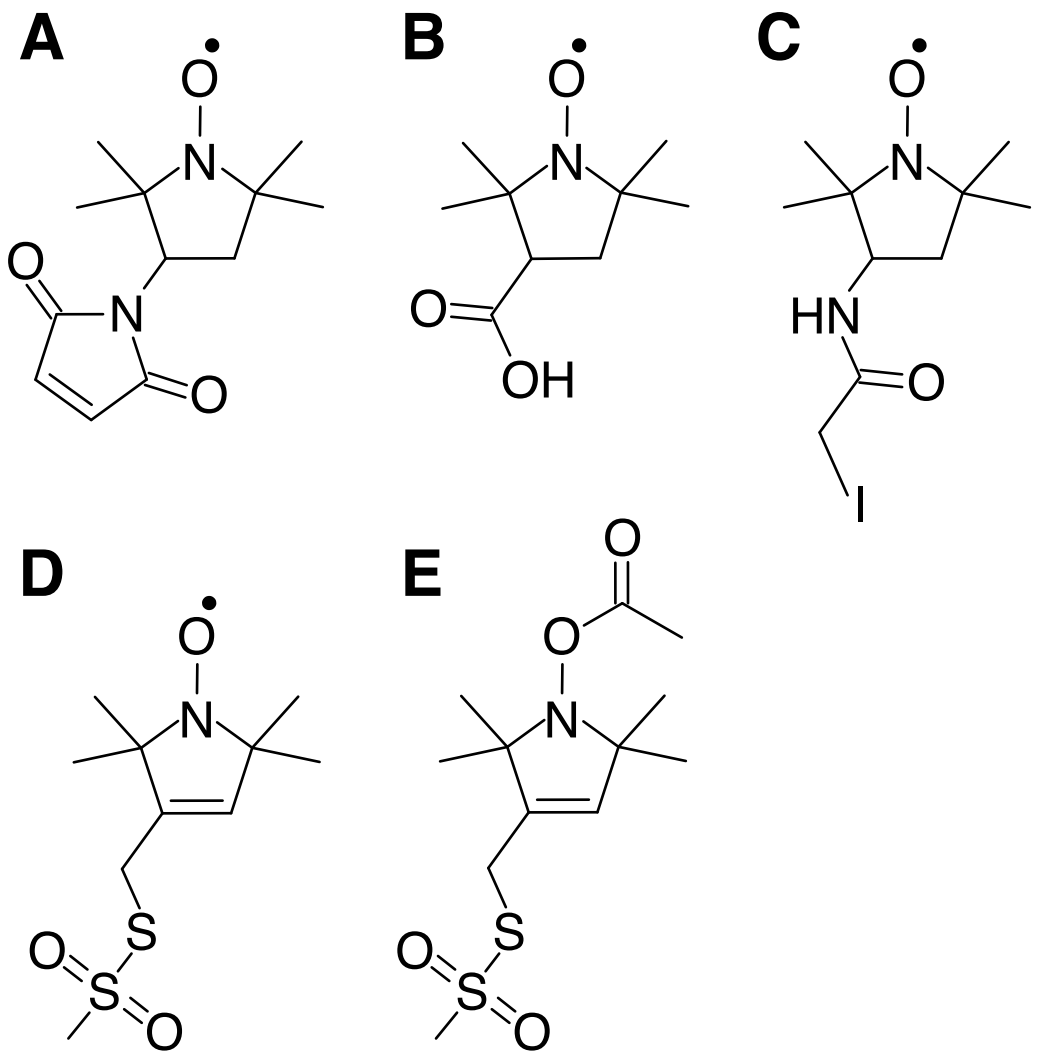

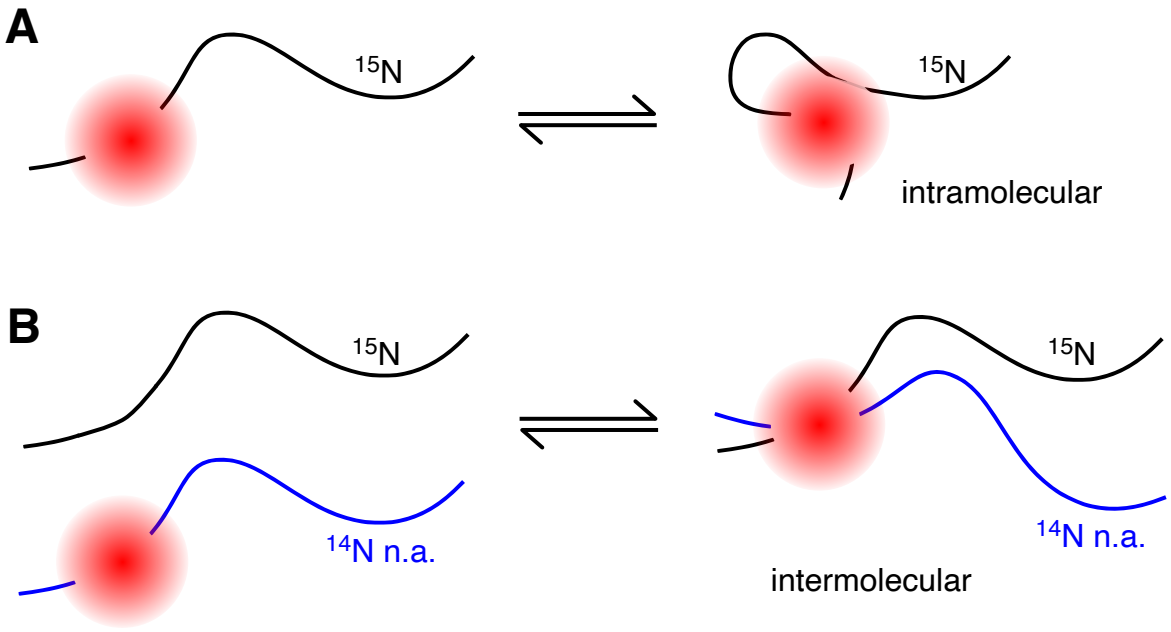

Figure

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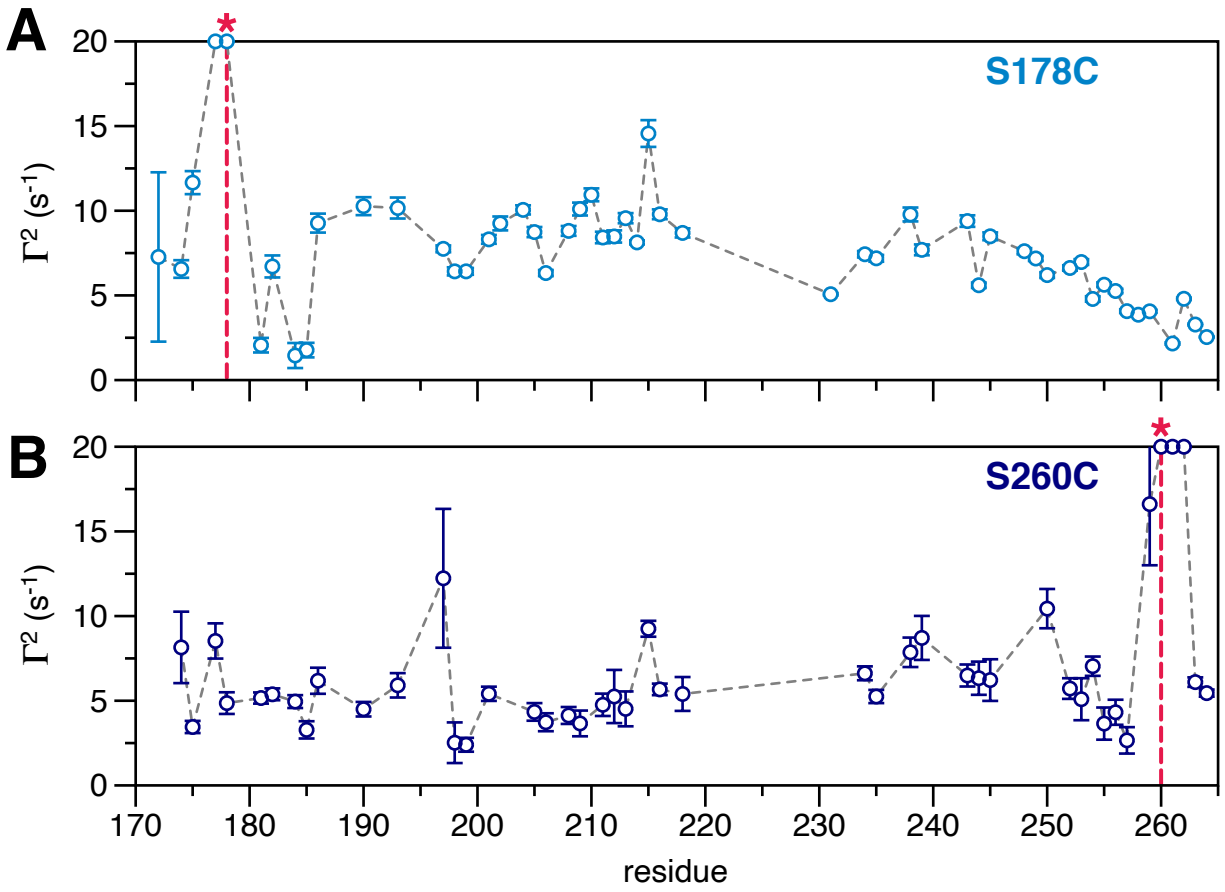

David S. Libich, Ph.D.
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July 30, 2021.

Amit Krishnan, Ph.D.
JoVE Review Editor
1 Alewife Center, Suite 200
Cambridge, MA 02140

Re: Authors Response to Reviewers of JoVE 63057.

Dear Dr. Krishnan,

Thank you for the opportunity to respond to the comments that the reviewers made regarding our recent JoVE submission "Paramagnetic Relaxation Enhancement for Detecting and Characterizing Self-Associations of Intrinsically Disordered Proteins." We have had a chance to review the comments and suggestions raised by the reviewers and have made the appropriate changes to the text and figures. We have also corrected the editorial comments noted by JoVE.

We sincerely thank the anonymous reviewers for spending their time and considerable effort reading and commenting on our manuscript. Incorporation of their suggestions has produced a clearer, more detailed, and most importantly, more accurate protocol. We have responded in detail to each point from each of the three reviewers in the appended pages.

Sincerely,

David S. Libich, Ph.D.

Assistant Professor, St. Baldrick's Foundation Scholar
Department of Biochemistry and Structural Biology
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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The overview by Johnson and Libich, presents an outline of a general protocol for the use of NMR paramagnetic relaxation enhancement (PRE) experiments to study intrinsically disordered proteins (IPSs). NMR is well-suited to the study of IDPs, as it is amenable to working in solution conditions and can provide information on several conformationally-relevant timescales which often involves very weak molecular interactions. Here, a protocol for the use of PREs to characterize conformational changes and intermolecular association is presented, which ostensibly can detect transient associations in the single-digit percentage population range. The protocol is detailed, although there is substantial room for the authors to add their own insights to help future readers. Additionally, while the representative results provide an example of the data analysis and processing, they do not guide the reader through the types of thorough quantitative analysis that is suggested in the introduction and discussion sections.

We appreciate the reviewer's time and effort in constructively criticizing our manuscript. We have addressed all of the points raised by the reviewer and made significant clarifications in the text. We welcome that the manuscript is clearer and more focused after these corrections. We address the reviewer's specific comments below.

Major Concerns:

Need to go over the types of thorough quantitative analysis that is suggested in the introduction and discussion sections.

We have clarified that we intended to position this protocol as an introductory method for casual and novice users to be able to obtain measurements of PREs on their intrinsically disordered protein (IDP) of interest. It is intended to provide the initial steps necessary to begin these types of experiments with a specific focus on IDPs. The reviewer is entirely correct that quantitative analysis of these types of data have been developed by several groups, but we note (and now describe) that the quantitative interpretation of IDP interactions using PREs can become complicated very quickly. We have made the decision to make the reader aware of the different approaches for analysis and have provided appropriate references to support further exploration of these analysis but have decided to limit the protocol to qualitative interpretations. We discuss some of the confounding factors that complicate qualitative interpretation to provide context to the complexity of these measurements. We have clarified these points in the Introduction, Protocol, Representative Results and Discussion sections.

Minor Concerns:

1. Line 57: "ridged" may be a typo for "rigid"

Corrected

2. Line 71-72: "detection" and "identification" are not quantifiable

Corrected – removed "to quantify"

3. Line 79: The PRE effect is also observed for systems having an anisotropic g-tensor; it is merely confounded by the presence of PCS and RDC that may make analysis more difficult (see ref 13 in this work).

We agree and thank you for pointing out this important point. We have clarified the text by adding: "*While nuclei with anisotropic g-tensors also produce a PRE effect, analysis of these systems is more difficult due to confounding effects contributed by the pseudo-contact shifts (PCS) or residual dipolar coupling (RDC)*."

4. Line 88: The conditions for detection of transient states by PRE should have a citation; eg: Anthis and Clore, Q. Rev. Biophys. 2015, 48(1), 35-116.

Citation was added

5. Line 105: The authors neglect other forms of metal chelated tags. If no room to list, then I'd suggest stating that there are a number of options for metal tags.

This is an astute comment, and we appreciate the work of many groups that have contributed to the development and characterization of tags useful for PRE and PCS measurements. We now note that there are many options for both the choice of the paramagnetic tags as well as for ligation chemistries. We chose to limit the discussion to nitroxide-based tags for their relative ease of use over metal-chelation tags since for example, ascorbic acid readily produces the diamagnetic condition while with metal-chelation tags, two samples are needed. We agree with the reviewer that there are multiple, good choices for PRE tags, and that the best choice for a particular protein is dependent on specific experimental considerations as well as the characteristics of the protein of interest. We have clarified this by adding: "*Several types of*

molecules have been proposed for use as spin labels including metal chelating (EDTA-based) and free-radical (nitroxide-based)." And have included additional references to direct the reader to alternatives.

6. Line 106-107: In addition to chemical linkage differences, nitroxide spin labels can be made more rigid through the introduction of bulky groups or the presence of a second linker.
Thanks to the reviewer for raising this important point. Certainly, one of the major challenges when deriving restraint information from PREs is accounting for the anisotropic motions of the paramagnetic tag. The complexity of this problem may be significantly reduced by using more rigid tags. We note that our protocol was intended for the specific case of detecting transient interactions between IDPs, and because of other unknowns introduced by flexible polypeptide chains, and the qualitative analysis presented, the rigidity of the tag is of lesser concern. We have noted this by adding: "*Inherent flexibility of the tag or of the linker may be problematic for certain analyses and in these situations, different strategies have been proposed to limit the motion of the tag such as by adding bulky chemical groups or the use of a second linker to anchor the tag to the protein (two site attachment).*" to the introduction and have included appropriate references.

7. Line 110-111: The choice of isotropic g-tensor also eliminates confounding RDCs in addition to PCS
Changed to "…has an isotropic *g*-tensor, no PCS or RDCs are induced…"

8. Line 115: move the comma to before "and"
Corrected

9. Section 1.1.1: Adding citations to the potential importance of native cysteines, or the deleterious effects of removing disulfide bridges would be helpful for future readers
This section was moved to the Discussion and expanded to describe how to deal with native cysteines as well as what might happen if native cysteines that participate in disulfide bridges are disrupted. Appropriate references were added.

10. Section 1.1.2: Why it is advantageous to introduce spin labels at multiple sites should be stated
This section was moved to the discussion and the explanation was expanded.

11. Sections 1.2.1 and 1.2.2 make more sense inverted, so that the note on expression system follows more closely to 1.2
Sections were re-ordered.

12. Section 1.2.3: This is protein, buffer, pH, and temperature dependent, and reducing agents may not be required at some or even all steps during purification depending on the system and even the engineered site within a protein. A citation and explanation on why this would be a requirement for IDPs would be useful.
The reviewer is correct to point out that need for including a reducing agent during all steps of the purification are dependent on several physiochemical factors as well as the characteristics of the protein of interest. We have found, that in the case of many self-associating IDPs, it is practical to always have reducing agent present. The text "*Preforming all purification steps in the presence of 1 mM reducing agent (DTT or TCEP) to prevent reaction of the free cysteine and formation of intermolecular disulfide bonds is recommended for IDPs although some systems may be more tolerant due to non-reducing conditions depending on the specific characteristics of the protein, as well as the pH, temperature and of buffer system chosen for purification.*" was added as well as a reference describing these points in more detail (Rabdano, et al., (2017) *Sci Rep*. **7**:11161)

13. Section 1.2.4: This should have an explanation for the case where no cysteine is present, and probably a citation as well
This was renumbered to section 1.2.3. We recommend removal of the purification tag in all situations, even if the tag is relatively simple and seems innocuous, (e.g. hexa-His tag) since the effect on the protein remains unknown in most situations. For example, for EWS 171-264, we realized that the presence of a hexahistidine tag alters the self-associative properties of the protein. The suggestion that the purification tag may contain a reactive cysteine was for a tag like GST or thioredoxin. The text has been revised to reflect this recommendation more clearly.

14. Section 1.2.5: This reference sample does not have a clear reference in the rest of the text.
This was renumbered to section 1.2.4. This is a recommended control sample to assess the occurrence of non-specific interactions between the tag and the protein of interest. Its utility is now described in the Discussion.

15. Line 257: I am a bit confused why you'd suggest a second delay estimate to be 1.2 times 15N T2. In most cases thew

15N T2 would be much longer than the 1H T2 (about 5x). Therefore 1.2 times 15N T2 delay in a proton T2 expt would result in signal that would be less than one percent of the original signal.
The reviewer is absolutely correct on this point and the approach to selecting an appropriate value for T_b was clarified to suggest starting at 1x the ^{1}H T_2 and optimizing from there by trading off S/N for accuracy in the T_2 measurement. Since many of the proteins we deal with are aggregation prone, they cannot be maintained at very high sample concentrations and thus it is important in these cases to optimize S/N for more reproducible sample-to-sample measurements. In general, PRE experiments are performed at low sample concentrations (< 300 μM) to limit the contribution from the solvent PRE effect.

16. Line 259-261: The 40-50% residual signal for high quality data is a bit misleading. This is a compromise between S/N and being able to sample longer T2 delay. The longer T2 delay will lead to more accurate T2 determination albeit with lower S/N. There is an optimal sampling delay.
We agree with the reviewer's point, and for the 2-point measurement approach, there is an optimal delay that accurately measures T_2 within a few percent of the T_2 values determined with a fully sampled evolution curve. In the qualitative case presented here, the absolute accuracy of the measured T_2 is less important than S/N for sample-to-sample reproducibility, thus the recommendation is to choose delays that do not lead to significant signal decay. Further, we note that there really is not an optimal T_2 delay for measuring both the paramagnetic and diamagnetic sample, thus common practice is to optimize S/N in the paramagnetic sample. We accept the reviewer's point and have included a clarification in section 5.1.

17. Section 2.2: Please provide a reference for not agitating during the labeling reaction, as this is commonly performed.
As the reviewer succinctly pointed out earlier, the exact incubation conditions are sample dependent, and since it is important not to introduce oxygen at this step vigorous agitation should be avoided. Gentle nutation or rocking may (or may not) improve the labeling reaction. The text has been clarified to reflect that nutation at this step may be helpful for the labeling reaction.

18. Line 216: "primary considerations" should be changed to "the primary consideration"
Corrected

19. Section 3.2.2. While the ratio of unlabeled and labeled protein should be optimized, this would strongly benefit by having a set of previously useful ratios from the authors, and the considerations they used for optimization.
Typical ^{14}N-labeled to ^{15}N-unlabeled protein concentrations were included as well as points to consider for optimization of the ratios are now included along with an appropriate reference (Fawzi et al., (2010) *PNAS*. **107**:1379).

20. Section 6.4: Define "significantly different" in the context of pulses
We have defined significantly different pulse lengths to be greater than a 0.5 μs difference on ^{1}H since differences larger than that will begin to manifest as errors when comparing peak intensities.

21. Section 7.1: It should be made clearer when certain programs substitute for others (eg: Sparky vs CCPN)
Thank you for pointing out this potential source of confusion. For the description of the protocol, we have decided to mention only Sparky to reduce the potential confusion of the reader. We now include a paragraph in the Discussion that details the many software packages available that can perform the analysis as described, their overlapping capabilities and now clarify where one package may be substituted for one another.

22. Line 305: the phrase "can be optimized" appears to continue from the previous sentence
Corrected

23. Section 8.2 refers to an NMRPipe command, and so should probably come before section 8.1, which states that the following steps will use Sparky
Corrected

24. Line 325: Purposeful exclusion of peaks seems unlikely, perhaps this should be changed to "unintentionally exclude peaks"
Corrected

25. Section 9.1: This is phrased strangely. "spreadsheet" should probably be altered to "spreadsheet software" and

"computing interpretative language" should be altered to "preferred programming language" and examples (eg: Microsoft Excel for the former, and python or MATLAB for the latter, should be provided).
Corrected

26. Section 9.5: This equation for uncertainty depends on the peak intensity and SNR, but the uncertainty is redefined in section 9.6 in a way that depends only on the root-sum-squared of 1/SNR. The equation in Section 9.5 is likely redundant.
Corrected - thank you for pointing out this oversight.

27. Line 352: "Observed Γ2 is indicative of residues that are in close proximity to the spin label" is either redundant with the sentence on lines 355-356 or missing a phrase.
Corrected

28. Lines 353-354: Please provide an estimate of the range where "close proximity to the spin label" leads to undetectable broadening.
Corrected – proximity range is now given.

29. Lines 356-357: Please expand on how qualitative interpretation of the data indicates that the protein is more compact than an extended chain.
The Representative Results and Discussion sections have been revised to address this point and points 32, 33 and 34 below. We have clarified the description of the results and provided more detail about what constitutes the observed PREs as well as what might be giving rise to these observations.

30. Line 357: delete "is" in "what is remains unknown"
Corrected

31. Line 360: "qualitatively" should probably be changed to "quantitatively"
Corrected

32. Line 361: Change "position" to "positions." This statement should be added to the earlier methods description where it mentions to prepare for alternative sites. Also, please expand on how more tags provides a "more accurate interpretation of specific interactions."
A statement was added at the beginning of the protocol to indicate that multiple mutants will be required to introduce spin-labels at different position in the protein. The discussion of the utility of multiple tags for interpretation of observed PREs was included in the Representative Results section.

33. In general, the Representative Results section could use careful attention to phrasing. Additionally, the introduction section of the manuscript seems to indicate that this protocol can be used for quantitative interpretation, but no quantitative results are included in this section. There is no clear indication to future readers about how the use of further tag sites would be beneficial to them, and no sufficient explanation of how the use of intra- and inter-molecular PREs would be beneficial in the presented example.
We have clarified the introduction to indicate that the presented method is aimed at casual or novice users of NMR to obtain PREs of their protein of interest. Further, we use IDPs as an example which, for reasons discussed in the text, make quantitative interpretation difficult in many cases. The readers are directed to the appropriate references that can significantly expand on the undelaying theory as well as quantitative analysis approaches that will build upon the introductory method presented. We have also rewritten the Representative Results section and expanded the Discussion sections to address several of the comments of the reviewers.

34. The discussion is well-written, and only serves to highlight some of the omissions in the earlier portions of the manuscript, as it includes references to "complimentary information" available from "placing the spin label at different positions within the protein" and the ability to differentiate intra- and inter- molecular sources of the observed PRE. Both of these are not well explored in earlier sections of the text, and would be very helpful if included in detail in the representative results.
The Discussion and Representative Results sections have been reworked to address these and other concerns raised by the reviewers.

Reviewer #2:

Manuscript Summary:

In this manuscript Johnson and Libich provide a protocol for NMR-based analysis of disordered proteins using paramagnetic relaxation enhancements obtained from covalent tags. The protocol manuscript is well-described and will be useful for a plethora of applications.

We thank the reviewer for their time and effort in reviewing our manuscript. We appreciate the constructive comments and are appreciative of the improvements in the communication of our protocol.

Some suggestions:

* Introduction: a short discussion and references to application highlights from recent literature would be great

We have added examples (with references) of studies where PREs were used to characterize alpha-synuclein forming fibrils, the self-association of FUS, as well as the interaction of two bacterial signaling proteins.

* Protocol, part 1: please expand the discussion of how the choice for positioning of spin labels should be made. What would be a good strategy to ensure that mutations don't affect (transient) structure and interactions? Things to come to my mind are CSPs, solvent PREs, but maybe the authors have some more ideas along this line.

This is an important consideration raised by the reviewer. We have expanded in both the Protocol and Discussion sections strategies to use for positioning spin labels, suitable residues to mutate, monitoring the effects of mutations, and how to deal with proteins that contain multiple cysteines.

* Protocol, buffers: please add that some buffers, i.e. phosphate, are not compatible with TCEP (short half-life)

Corrected – added a sentence to clarify that compatibility between buffer components should be considered and that some combinations should be avoided outright. A reference was included to reinforce this point. (Burns et al., (1991) *J Org Chem.* **56**:2648).

* Line 194: completeness of SL incorporation of the 15N-labeled protein can also be monitored by looking at the HSQC cross peak of the tagged residue which should disappear completely if fully tagged.

We appreciate the excellent suggestion by the reviewer. As noted by other reviewers, the use of mass spectroscopy to judge label incorporation is problematic for several reasons. This suggestion has been incorporated.

* If there is space, the authors could mention determination of PREs using 13C/15N labeled samples. This can provide additional PREs of 13C-bound protons and allow recording of triple resonance experiments to reduce overlap (e.g. HNCO in combination with NUS). For IDPs this has been proven useful. Detection of 13C PREs using 13C direct detection has been reported as well.

Thank you to the reviewer for pointing these additional approached to measuring PREs. We are aware of the use of the HNCO experiment as a way of overcoming the dispersion problem for IDPs. As an aside, we have utilized the HNCO approach, and we found that in the case of proteins with repetitive sequences (like the example in the manuscript) the gains in resolving certain residues were not always consummate with the extra effort required for protein preparation for example. Thus, for this article that is aimed at for a casual user or novice audience, we opted to keep the approach as simple as possible. We suggest increasing acquisition time (where appropriate for an IDP) to help resolve partially overlapped peaks and describe a simplified quantitative analysis. We have added a paragraph in the Discussion section that describes the use of HNCO experiments and measuring ^{13}C PREs that would be useful in many cases.

* Line 244: we prefer to calibrate the 15N pulse using the same sample. This can be done using a HMQC-based experiment where the length of one of the 90° 15N pulses is varied (loss of signal -> 180°) to calibrate the 15N 90° pulse.

We agree with the reviewer that this is the most accurate way of calibrating the 90º ^{15}N hard pulse. We do note however, that for the two-point measurement of $^{1}H_N$ T_2 and qualitative analysis described in this manuscript, using an external standard provides an accurate enough calibration for the ^{15}N pulse length. We have included the reviewer's suggestion and added a sentence in the Protocol describing alternate approaches for calibrating nitrogen pulse lengths.

* Line 345: please check the error propagation formula. It should contain the partial derivatives of the formula shown in 9.3 in the peak intensities

Corrected - thank you for pointing out this oversight.

* Line 355: the PRE depends on the distance, population and the dynamics (tau_c) of the interaction vector connecting the electron and the nuclear spin.

This was clarified in the Representative Results section with the following sentence: "*In the case of EWSR1 171-264, attributing the source of the PRE effect is complicated since it may arise from a combination of inter and intra residue contacts, and is dependent on the distance from the nucleus to the paramagnetic center, the population of that conformation, and the dynamics of the vector connecting the electron and nuclear spins.*"

* The inter-molecular PRE depends on the protein concentration (-> population), whereas the intra-molecular PRE does not (assuming that correlation times don't change with concentration etc.). This might need to be included, together with a statement that the Kd and dynamics of the inter-molecular interactions affect the inter-molecular PRE.
Thank you to the reviewer for this comment. These are important points that needed clarification. Lines were added to both the Representative Results and Discussion sections to expand and reinforce these concepts.

Reviewer #3:
Manuscript Summary:
The protocol from Johnson and Libich provides some recipes for measuring PREs. The protocol reflects standard practices and advice on how to design the experiments, as well as a protocol using scripts from NMRpipe and Sparky.
Thank you to the reviewer for their time and effort in reviewing the manuscript. We appreciate their comments and critiques, and that the manuscript is strengthened because of their input.

Major Concerns:
The authors recommend 3-maleimido-PROXYL but in the list of reagents they just mention MTSL and the reference they provide (81213-52-7) is not the supplier reference but the CAS (Chemical Abstract Service) registry number.
Corrected

Figure 1 legend contains various errors: the structures depicted include radicals and diamagnetic molecules (e); a variety of derivatization chemistries, not just maleimido; entries (d) and (e) are pyrroline -with the unsaturated ring- and entries (a)-(c) are pyrrolidine -with the saturated ring- the rigidity of the ring is different. The substituted pyrrolidine has a chiral center and, presumably, the commercial product is racemic, which will lead to a mixture of diastereomerisomeric paramagnetic proteins. Not too serious an objection for the qualitative analysis described but, considering this is a protocol, the reader should be warned not just about the problem of pseudocontact shifts -not present- but also on the stereochemical heterogeneity, that would be present, although probably not noticed.
Thank you to the reviewer for pointing this out, and we agree that while these effects will be small for most cases, there is the potential for producing unintended results. The following text and references were added to clarify this point: "*Inherent flexibility of the tag or of the linker may be problematic for certain analyses and in these situations, different strategies have been proposed to limit the motion of the tag such as by adding bulky chemical groups or the use of a second linker to anchor the tag to the protein (two site attachment)* (Lindfors et al., (2008) *J Biomol NMR*. **3**:157). *Additionally, commercially available tags may contain diastereomeric proteins but generally this will not contribute to the observed PRE* (Bleicken et al., (2019) *ChemistryOpen.* **8**:1035)." The errors in the Figure 1 caption were corrected.

Minor Concerns:
The error analysis presented in part 9 is obscure. The same symbol (sigma) is used for inequivalent errors (peak intensities and relaxation rates). A clearer explanation is given in reference 22.
Corrected - thank you for pointing out this oversight.

The problem with using mass-spectrometry for quantification of the level of incorporation is more fundamental than the possible difficulties of some proteins to be ionized.
We agree with the reviewer and note that this was pointed out by other reviewers as well. We have removed the recommendation to monitor label incorporation by mass spectroscopy and replaced it with the more straight forward method of tracking labeled residue peak intensity. We note the limitation of this approach for some IDPs for example if the peak is overlapped, and thus also suggest the use of Ellman's reagent.

Some typing errors should be corrected (e.g. Line 394 Complementary)
Corrected

Editorial comments:

Editorial Changes

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.

Corrected

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Corrected

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

For example: TopSpin, MilliporeSigma, Bruker, New Era, etc.

Corrected. We note that the instructions and pulseprograms described are written specifically for Bruker spectrometers running TopSpin version 3.2 or later.

4. 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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Corrected

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Corrected

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Corrected

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Corrected

8. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Appropriate protocol steps that should be visualized are highlighted.

9. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Corrected

10. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.
Corrected. Note that these errors arose from the JoVE EndNote style template that was downloaded from the JoVE website. We were using Endnote 19 and Mac OSX 10.15.X and the issue was replicated on multiple computers. Upon further inspection of the template file, the “&” modifier is included in the “*Bibliography:Author Lists*” field in the output style.

11. Please use uppercase letters to label the figures.
Corrected

12. Please sort the Table of Materials in alphabetical order.
Corrected