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When two become one: Integrating FRET and EPR into one structural model

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In modern structural biology, rarely one method can provide all structural insights, and integrative approaches combining different results into one framework are the necessary future. For example, cryo-electron microscopy/tomography and x-ray crystallography are well-established techniques yielding atomic-resolution structures of folded proteins. However, these types of structural information may be limited in providing insights on highly dynamic processes or intrinsically disordered proteins. Such data can be complemented by other classes of techniques, such as single-molecule fluorescence resonance energy transfer (smFRET) and electron paramagnetic resonance (EPR) spectroscopy, which measure distances or even distance distributions between labels that can be site-specifically mounted into the biomolecule.

FRET refers to the nonradiative energy transfer between an excited-state donor fluorophore (dye) and an acceptor. The energy transfer relies on dipole-dipole coupling mechanisms, and its efficiency depends on the sixth power of the distance between the dyes as well as their relative orientation. FRET is an attractive tool as a "molecular ruler" in the range of 3–10 nm. The dependence on the relative dye orientation often adds uncertainty and can be addressed via long linkers, which ensure the rotational freedom of the dyes, allowing us to use a constant ($\kappa^2 = 2/3$) as orientation factor. Although obtaining accurate distances from FRET efficiencies can be relatively complicated, as various photophysical effects need to be accounted for and sample-specific calibrations may be required, recent community-wide studies show high precision and reproducibility across laboratories (1,2). FRET offers numerous strengths such as the ability to be carried out at ambient temperatures and at the single-molecule level in vitro and in vivo, thereby giving information on distances and structural dynamics in physiologically relevant conditions and beyond the ensemble average.

EPR, using double electron-electron resonance (DEER), measures distance-dependent electron dipolar spin-spin coupling and can report not only on the average distances between two or more paramagnetic centers but also on the shape of the distance distributions (3). The range probed can lie between 1.5 and 16 nm. Immobilization at ambient temperature or shock freezing and measurement at cryogenic temperatures (10–50 K) are often required for high-precision DEER. In EPR, the calculation of distance distributions relies on relatively well-established analysis methods, requires no calibration, and the relative orientation of the spin labels can mostly be neglected.

For both smFRET and EPR, the conversion of distance constraints between labels into absolute and accurate distance information of the actual protein backbone chain requires that the labels are taken into account explicitly. In fact, converting from an accurately measured FRET distance between the dye centers to the actual distance between the residues of the protein chain is nontrivial. In EPR, spin labels are generally much smaller and can accommodate shorter linkers compared with fluorescent labels. This has facilitated the development of various modeling approaches for consideration of labels in EPR. One standard approach is the use of rotamer libraries, which are libraries containing representative conformers (rotamers) of the label, covering its entire conformational space (4). For each specific labeling site, the ensemble of conformers that satisfies the local steric constraints can be calculated. This procedure is significantly more computationally efficient than the direct calculation of the label conformations on the protein. Notably, rotamer libraries can predict anisotropic conformations of the spin labels (5). The widespread usage of similar libraries for FRET has not been feasible because of the generally larger size of fluorescent labels and their

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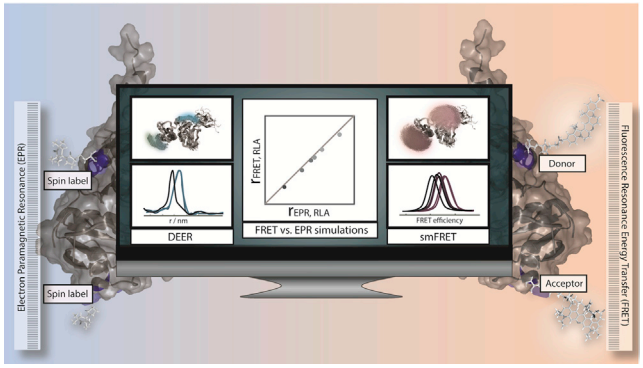

FIGURE 1 EPR (*left*, *blue*) and FRET (*right*, *orange*) both report on distances between spin labels or donor-acceptor pairs attached to the protein. Using a rotamer library analysis (RLA), the conformational distributions of the label on the protein were computed for EPR and FRET. The correlation between the distance distributions obtained from RLA is shown in the center plot, validating that RLA can integrate FRET and EPR into one framework.

linkers. Instead, the commonly used approach calculates the accessible volume of the labels by taking into account the linker length/width and dye radii (6).

The lack of a common framework in this aspect has been a major obstacle in the combination of smFRET and DEER spectroscopy results. Because these techniques are complementary, such a combination would be very valuable for integrative structure determination.

To tackle this problem and establish a joint workflow for the integration of smFRET and EPR spectroscopy, in this issue of *Biophysical Journal*, Klose et al. (7) set out to extend the rotamer library analysis (RLA) from EPR to smFRET and validate their results experimentally. They selected a construct composed of two rigid RNA recognition motifs (RRM3/4) from a well-structured protein, human polypyrimidine tract binding protein, and designed six variants for labeling with either spin or fluorescent labels. All selected residues lied at solvent-exposed regions, some distances only differing by one α-helical turn . One α-helix turn is 5.4 Å, so it is quite a demanding case.

To generate the rotamer libraries, they first performed the geometry optimization of the fluorescent labels (Cy3b and CF660R) with linkers and cysteine side chains, using quantum chemistry calculations. Then, they generated Monte Carlo assemblies of the labels and clustered the resulting ensemble to generate the final rotamer library composed of representative rotamers. They combined the library with the RRM3/4 structure to calculate interlabel distances and then converted these to FRET efficiencies, taking into account possible sources of uncertainty, e.g., of the Förster radius (R_0) and the diffusional motion of the fluorophores. Experimental FRET efficiencies were obtained by smFRET measurements of purified and labeled RRM3/4 protein constructs. The results were also evaluated using accessible volume simulations. For EPR, the distance distributions obtained from DEER measurements were compared with the distance distributions obtained from RLA. The study evaluated the performance of RLA for FRET against the well-established EPR RLA, while simultaneously probing the limits of both methods. The two α-helices in RRM3/4 provided a suitable construct to resolve well-defined variations using FRET and EPR. The work and main results are summarized in Fig. 1.

The results are impressive, underlining the usefulness of rotamer library generation for FRET, the sensitivity of both methods, and the agreement between the results. A precision of 0.25 nm found in many cases is remarkable, considering that the size of the fluorophore labels is considerably larger than the distance variations being probed (easily ~1 nm or more for many dyes). This high resolution makes EPR and smFRET ideally suited for modeling and probing minute conformational changes at a high level of precision.

The resolution limits reported in this study do not surpass previously reported ones; especially in FRET, uncertainty sources due to dye photophysics and local environment continue to be an obstacle. Furthermore, the generation of rotamer libraries for FRET requires the use of correction factors, which may need optimization with experimental data when changing the study system. Nevertheless, the potential advantages vastly outweigh the drawbacks. smFRET and EPR are placed under the same analysis framework, thereby facilitating the integration of their results. The precise distance distributions obtained by EPR at cryogenic temperatures can be combined with the distances obtained with ambient temperature and single-molecule level FRET measurements, promising exciting new findings and more complete models in integrative structural biology.

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