Supplementary Material (ESI) for Chemical Communications


**Electronic Supplementary Information**

# Simultaneous molecular imaging based on electron paramagnetic resonance of $^{14}$N- and $^{15}$N-labelled nitroxyl radicals

Anna Pawlak,[a] Ryohei Ito,[a] Hirotada Fujii,[b] and Hiroshi Hirata[a]

[a] Division of Bioengineering and Bioinformatics, Graduate School of Information Science and Technology, Hokkaido University, North 14, West 9, Kita-ku, Sapporo 060-0814, Japan

[b] Department of Arts and Sciences, Centre for Medical Education, Sapporo Medical University, South 1, West 17, Chio-ku, Sapporo 060-8556, Japan

**Contents**



## *Materials and Methods*

### Separation of spectra

In simultaneous electron paramagnetic resonance (EPR) imaging of $^{14}$N- and $^{15}$N-labelled nitroxyl radicals, one of the major issues regarding image reconstruction is the overlapping of EPR absorption peaks, which results from the application of magnetic field gradients, necessary for encoding the spatial distribution of unpaired electron spins into EPR spectra. $^{14}$N- and $^{15}$N-labelled nitroxyl radicals have three-line and two-line absorption spectra, respectively with each absorption peak clearly separated when no magnetic field gradient is applied (see Supplementary Figure S1). However, the distortion of the line-shapes and overlapping of EPR absorption peaks that occurs in the presence of magnetic field gradients

means a method of spectral separation is required for the simultaneous EPR imaging of $^{14}$N- and $^{15}$N-labelled nitroxyl radicals. Since our EPR spectrometer has been based on the continuous-wave protocol, which uses a constant microwave frequency and sweeping of the external static magnetic field, the EPR spectra we measure are recorded as a function of the external static magnetic field.

The process of spectral separation of $^{14}$N- and $^{15}$N-labled nitroxyl radicals was carried out by subtracting the spectrum of $^{14}$N-labled nitroxyl radicals from overlapping spectra. Since the line-shapes of the absorption peaks of three-line EPR spectrum are similar to each other, shifting the line-shape of the center peak could approximate that in the lower field. To shift the measured center peak to an arbitrary position in the magnetic field, we used the shifting theorem of Fourier transform. If the center peak of an EPR spectrum of $^{14}$N-labelled nitroxyl radicals is given by the function $f$ as a function of magnetic field $B$, the shifted line-shape $f_{\text{shifted}}$ is expressed by

$$f_{\text{shifted}}(B) = \text{IFT}\left\{\alpha \sum_{n=0}^{m} c_n \exp(-i\omega_n \gamma)\right\}, \quad (1)$$

where $c_n$ is $n$th complex Fourier coefficient of the line-shape of the center peak, IFT means inverse Fourier transform, $i$ is the imaginary unit, $\omega_n$ is $n$th spatial angular frequency. Parameters, $\alpha$ and $\gamma$ were used to adjust the amplitude and the position of the approximated line-shape, respectively. For the zero-gradient spectra of $^{14}$N- and $^{15}$N-labelled nitroxyl radicals, the parameters $\alpha$ and $\gamma$ were determined by searching the minimum value of the root mean square of the residual EPR absorption signal, which was calculated by subtracting the approximated line-shape given in Equation (1) from the measured, overlapping spectra. By using those parameters, the signals of $^{14}$N-labelled nitroxyl radicals were subtracted from lower half of each projection.

## Chemicals

4-Hydroxyl-2,2,6,6-tetramethylpiperidine-$d_{17}$-1-$^{15}$N-1-oxyl (TEMPOL-$d_{17}$-$^{15}$N) and 4-hydroxyl-2,2,6,6-tetramethylpiperidine-$d_{17}$-1-oxyl (TEMPOL-$d_{17}$-$^{14}$N) were purchased from CDN Isotopes Inc. (Quebec, Canada). Hydroxylmethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (HMP) was obtained from Toronto Research Chemicals Inc.(North York, Canada). All nitroxyl radical solutions were prepared in phosphate-buffered saline (PBS).

Nuclear-localising spin-probe, F-DisT, was a gift from Dr. Hidehiko Nakagawa, Nagoya City University, Japan.[1] F-DisT solution was prepared in a mixture of dimethyl sulfoxide (DMSO) 0.1 %, PBS 89.9%, and ethanol 10% (v/v).

## Phantom

In two-dimensional (2D) EPR imaging, six capillary tubes (inner diameter of 1.9 mm and outer diameter of 2.5 mm) were held with a bobbin (22 mm in diameter and 25 mm long) made of cross-linked polystyrene, Rexolite® 1422. Each capillary tube contained 0.1 mL of solutions (35 mm long in tubes) of nitroxyl radicals. Capillary tubes were filled with TEMPOL-$d_{17}$-$^{15}$N and/or TEMPOL-$d_{17}$ in distilled water. In three-dimensional (3D) EPR imaging, two capillary tubes were filled with the solution of 1 mM TEMPOL-$d_{17}$-$^{15}$N and three capillary tubes were filled with the solution of 1 mM TEMPOL-$d_{17}$. Another capillary tube was filled with distilled water. Each capillary tube was filled with 50 μL of the solutions (17.5 mm long in tubes).

## Image reconstruction

Measured EPR spectra with and without magnetic field gradients were divided into two parts, a lower half (1-256 data points) and a higher half (257-512 data points). The 256-point data arrays were transferred to the 512-point data arrays by zero-filling method. EPR spectra

for $^{15}$N-labelled nitroxyl radicals were recovered by subtracting the signal of $^{14}$N-labelled nitroxyl radicals. To precisely perform the subtraction in the spectra, we obtained the parameters $\alpha$ and $\gamma$ in Equation (1), for approximating the EPR absorption peak in the lower field. A scheme of data processing is illustrated in Supplementary Figure S1. We used the method of filtered-back projection for image reconstruction with a Hamming window function. Surface-rendered images were drawn by IDL 7.1 data visualization software (ITT Visual Information Solution, Boulder, CO).

**Animal preparation**

The protocol for all animal experiments was approved by Sapporo Medical University Animal Care Committee (approved no. 08-081) according to the National Institute of Health Animal Care and Use Protocol (NIH, Bethesda, MD, USA). Male ICR mice were supplied from Japan SLC Inc. (Shizuoka, Japan) and were 6 to 7 weeks of age at the time of EPR experimentation. Mice were housed three per cage in climate-controlled, circadian rhythm-adjusted rooms and were allowed food and water *ad libitum*. Mice were anesthetized with isoflurane (1.5%) in air of 250 mL/min, and were set on the bed for imager. The tail vein was cannulated with 26G needle for the injection of nitroxyl radicals in PBS. A solution of nitroxyl radicals in PBS, 0.7 μmol/g weight of body, was injected via tail vein cannulation for 30 s duration.

**EPR imaging**

An in-house built 750-MHz continuous-wave (CW) EPR imager was used in the experiments with phantoms. The details of our 750-MHz CW-EPR imager have been described elsewhere.[2,3] Only the outline of the EPR imager is described here. The main magnet has a magnetic field of 27 mT, and its magnetic circuit is formed with permanent

magnets (X-5253, NEOMAX Company, part of Hitachi Metals, Tokyo, Japan). The main magnet was used with three pairs of coils for field gradients and a single pair of coils for magnetic field scanning. A multi-coil parallel-gap resonator was used in the bridge.[4] The sample space of the resonator was 22 mm in diameter and 30 mm in length. The unloaded quality factor and the efficiency for generating an RF magnetic field were 540 and 43 $\mu T/W^{1/2}$, respectively. To control data-acquisition in EPR imaging, we used National Instruments LabVIEW 8.5 on MacOS 10.5 and an Apple MacPro computer.

For 2D phantom imaging of TEMPOL-$d_{17}$-$^{14}N$ and TEMPOL-$d_{17}$-$^{15}N$ solutions, the measurement parameters were as follows: RF power 2.5 mW, field scanning 3.0 mT, magnetic field modulation 0.06 mT, field gradient 40 mT/m, duration of field scanning 1.0 s, time constant of lock-in amplifier 1 ms, number of projections 64, and number of averages 2. The total acquisition time was 2 min 41 sec. The results of the spectral separation for the measured projections are given in Supplementary Figure S2. These spectra were measured in 2D phantom imaging.

For 3D phantom imaging, the measurement parameters were as follows: RF power 2.5 mW, field scanning 3.0 mT, magnetic field modulation 0.06 mT, field gradient 40 mT/m, duration of field scanning 1.0 s, time constant of lock-in amplifier 1 ms, number of projections 126, and number of averages 2. The total acquisition time was 5 min 13 sec.

Immediately after the injection, the subject mouse was placed in the EPR imager, and the imager was adjusted to scan 3D images for the distributions of HMP and TEMPOL-$d_{17}$-$^{15}N$ radicals in the mouse head. For animal imaging, the measurement parameters were as follows: RF power 3.2 mW, field scanning 3.0 mT, magnetic field modulation 0.1 mT, field gradient 20 mT/m, duration of field scanning 0.4 s, time constant of lock-in amplifier 1 ms, and number of projections 46. The total acquisition time was 23 s for a single 3D image scan.

**EPR spectroscopy of nucleus-localising spin probe F-DisT**

Line-shapes of nitroxyl radical may be influenced by its microenvironment if the radical is attached to a bigger molecule. To examine a spin labelled target molecule, we measured a nucleus-localizing spin probe, F-DisT. For EPR spectroscopy of a solution of 0.5 mM F-DisT, the measurement parameters were as follows: RF power 2.5 mW, field scanning 6.0 mT, magnetic field modulation 0.1 mT, duration of field scanning 2.0 s, time constant of lock-in amplifier 1 ms, and number of averages 200. The total acquisition time was 8 min 12 sec.

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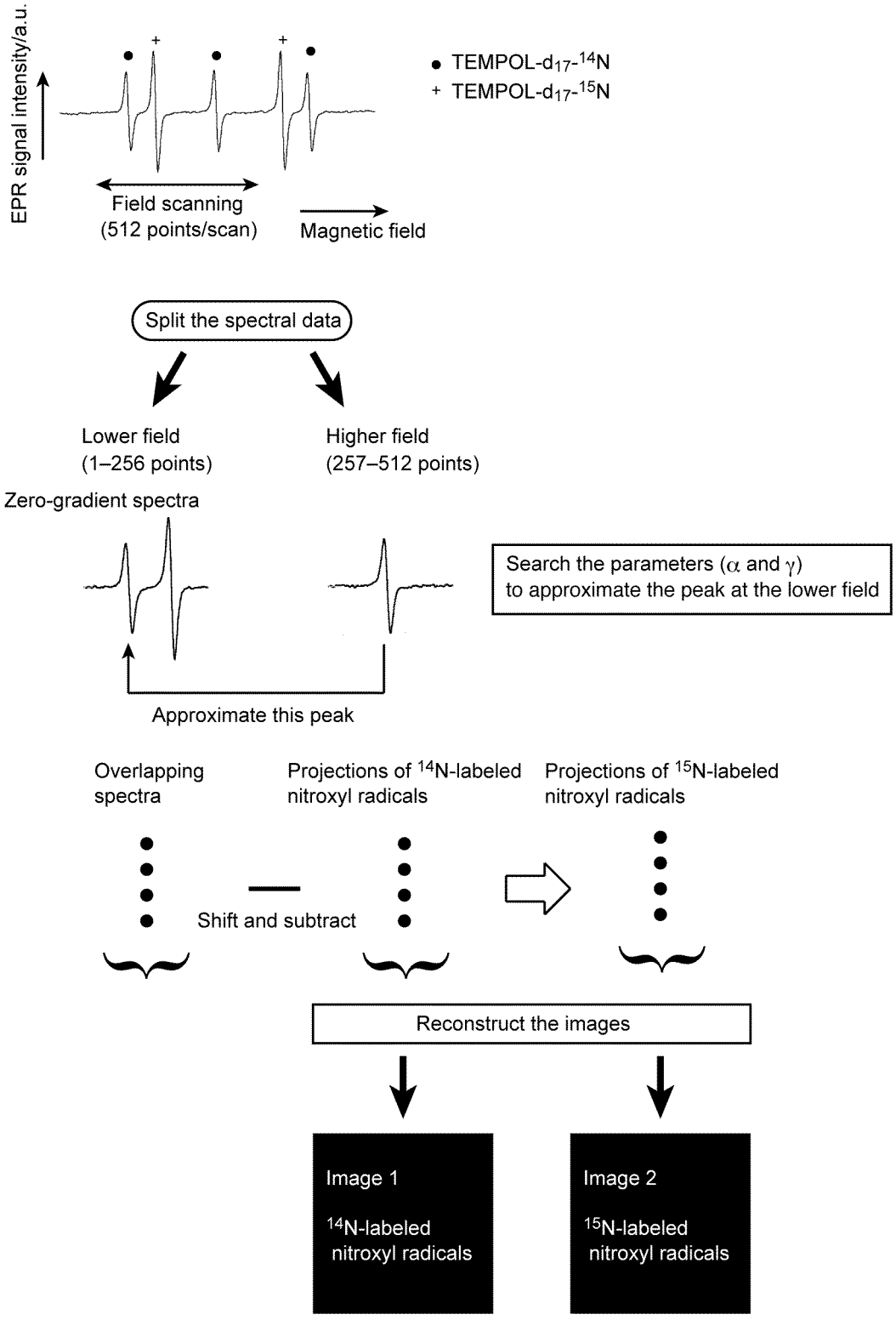
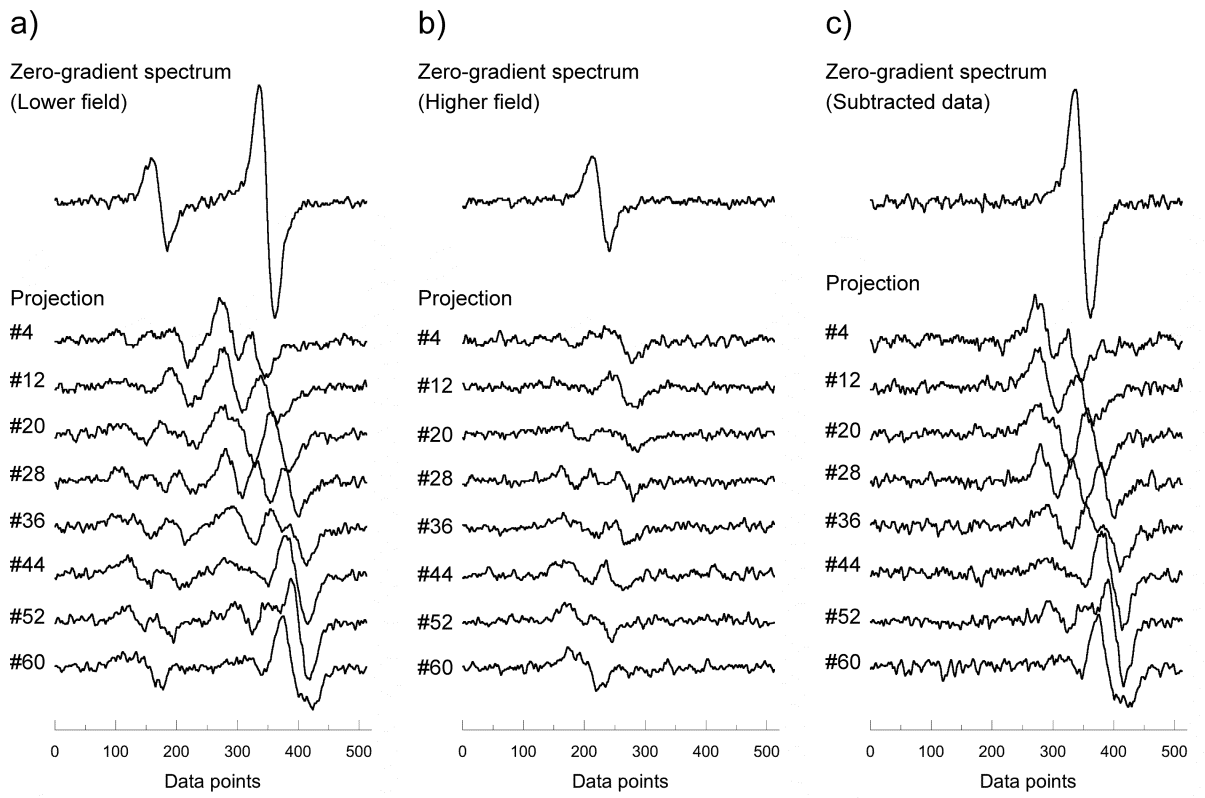
2 H. Akaba-Sato, H. Fujii, H. Hirata, *Rev. Sci. Instrum*. 2008, **79**, 123701.

3 H. Akaba-Sato, Y. Kuwahara, H. Fujii, H. Hirata, *Anal. Chem*. 2009, **81**, 7501–7506.

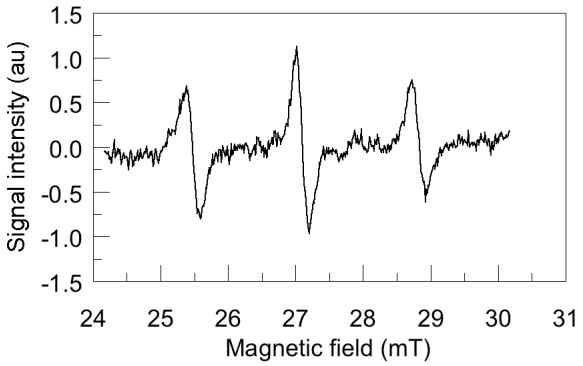
4 Y. Kawada, H. Hirata, F. Fujii, *J. Magn. Reson*. 2005, **184**, 29–38.




Supplementary Figure S1
Concept of spectral separation for $^{14}$N- and $^{15}$N-labelled nitroxyl radicals.




Supplementary Figure S2
Zero-gradient EPR spectra and projections under magnetic field gradients measured in 2D EPR imaging for TEMPOL-$d_{17}$ and TEMPOL-$d_{17}$-$^{15}$N radicals. a) EPR spectra in the lower field, b) EPR spectra in the higher field, and c) subtracted EPR spectra in the lower field. Projections in b) were due to TEMPOL-$d_{17}$ radicals, and projections in c) were due to TEMPOL-$d_{17}$-$^{15}$N radicals. Data of each spectrum (256 points) were converted into the data arrays of 512-points with the zero-filling method.




Supplementary Figure S3
EPR spectrum for nucleus-localising spin probe (0.5 mM F-DisT solution, 0.4 mL). No significant symmetry loss of the EPR spectrum was observed. However, little difference in the signal intensities and line-widths of the lower and center fields were detected.