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Hot article: New method for mixture analysis

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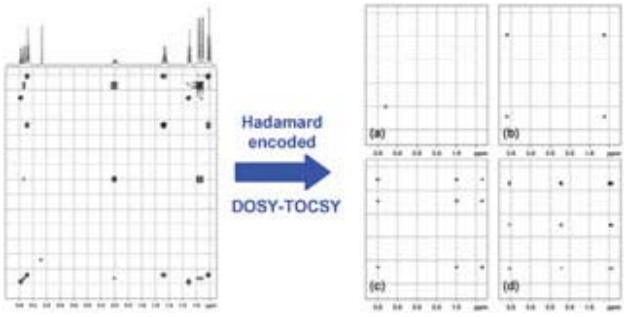
Stéphane Viel and Stefano Caldarelli, at the University of Aix-Marseille, France, used diffusion ordered NMR spectroscopy (DOSY) to analyse mixtures of compounds. DOSY is a technique that discriminates between compounds according to their size and shape. The NMR spectrum of a complex mixture can be broken down into the spectra of its components, making structural assignment much easier. The best performance is achieved when the spectra are recorded at two or more different frequencies but this takes a number of hours to do. However, Viel developed a new method that allows the experiments to be completed in minutes.



'This work is related to a series of studies that have been conducted in our, as well as other, research groups, with the aim of improving DOSY,' said Viel. 'Although a large amount of work must still be accomplished, it is believed that the present investigation will contribute to this global process and inspire future exciting developments.'

Joanne Thomson

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Improved 3D DOSY-TOCSY experiment for mixture analysis
Stéphane Viel and Stefano Caldarelli, *Chem. Commun.*, 2008, 2013
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Improved 3D DOSY-TOCSY experiment for mixture analysis†

Stéphane Viel* and Stefano Caldarelli

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With respect to best currently available pulse sequences, a 10-fold reduction in minimum experiment time together with significant resolution enhancement can be achieved in 3D DOSY homonuclear experiments by means of Hadamard encoding, as illustrated here for the 3D DOSY-TOCSY experiment.

Analysis of complex mixtures currently represents an active field of research. In this context, a commonly used analytical method is hyphenation, *i.e.* the sequential combination of chromatography with single or multiple spectroscopic techniques such as UV, mass, or NMR spectrometry.[1] Impressive applications of hyphenated and related techniques on a large variety of complex matrices have already been reported.[2–4] Alternatively, diffusion ordered NMR spectroscopy (DOSY) appears as an attractive technique for mixture analysis because it allows substances to be discriminated according to their molecular size[5,6] and, for isomers, according to their molecular shape.[7] Unlike hyphenated techniques such as, for instance, LC-NMR, which require specific instrumentation, DOSY can be easily implemented on routinely available NMR instruments, the only prerequisite being the availability of a gradient coil and amplifier, which both accompany nowadays most high resolution NMR spectrometers. Basically, DOSY requires ^{1}H spectra to be recorded at increasing diffusional attenuation, resulting for a given frequency in an exponential decay that must be inverted to yield its corresponding diffusion coefficient (D). A pseudo 2D spectrum can then be constructed, showing NMR chemical shifts on one dimension and diffusion coefficients on the other. The NMR spectra of the mixture components can thus be resolved, thereby easing the overall structural assignment without the need for any preliminary physical separation.

The data inversion step undeniably represents the key to DOSY. When the resonances are completely separated in the frequency dimension, high resolution in the diffusion dimension can be achieved with a simple two-parameter monoexponential fitting, an approach referred to as high resolution DOSY.[8] In contrast, when significant spectral overlap occurs, the methodology fails. In this case, unless the diffusion coefficients of the overlapping resonances differ significantly, incorrect D values will be obtained, especially in the presence of noise. Without *a priori* knowledge of the system, this data processing step may end up in an intractable problem.[5] As emphasized by Nilsson and Morris,[9] two main strategies can hence be adopted to circumvent this difficulty. The first relies on sophisticated post-processing and data analysis schemes,[5,10] which may lead to significant resolution enhancement in the diffusion dimension.[11,12] The second consists of simplifying the data analysis by minimizing signal overlap. This is classically achieved through additional frequency dimensions by combining a diffusion encoding step with a 2D correlation building block, which can split a crowded spectral region into distinct sub regions on which exponential fitting procedures can be subsequently applied. This has resulted in numerous 3D DOSY pulse sequences.[5,9,13–18] Another promising technique was independently proposed by two research groups,[19,20] who used conceptually different methods to suppress multiplet fine structures to achieve simplified homodecoupled ^{1}H spectra. Although a significant loss in sensitivity must be deplored in both cases, exciting developments may already be anticipated.

Typically, bringing an additional frequency dimension to DOSY experiments leads to a considerable increase in experimental time, and hence a few methods were proposed to minimize this time penalty. Vitorge and Jeannerat suggested computer optimized spectral aliasing of the indirect dimension in ^{1}H–^{13}C HSQC experiments in order to profit as much as possible from the gain in resolution afforded by ^{13}C spectra.[21] Morris and coworkers proposed a series of efficient pulse sequences in which the diffusion-encoding step is internally incorporated into the 2D correlation block, giving rise to the so-called[9] IDOSY pulse sequences family (HMQC-IDOSY,[14] 2J-IDOSY,[9] COSY-IDOSY,[16] DQFCOSY-IDOSY[18]). In this case, the time saving is mostly achieved through phase cycling reduction. Another method used for minimizing experimental time relies on Hadamard-encoded NMR spectroscopy, which has been extensively described by Freeman and coworkers,[22] and where the evolution time in the indirect dimension of the 2D block is replaced by phase-encoded multisite selective excitation, in order to focus the experimental time on the signal-containing spectral regions. Steinbeck and Chmelka demonstrated the use of *heteronuclear* Hadamard-encoded NMR diffusion measurements to allow overlapping signal decays to be resolved with substantially shorter measuring times.[23] Interestingly, however, no application to *homonuclear* 3D DOSY experiments has been reported so far, although the experimental time saving offered by Hadamard encoding in homonuclear 2D correlations has long been realized.[22]

Aix-Marseille Université, ISM2-UMR-6263, Equipe CES, Campus de Saint Jérôme, case 512, Av. Escadrille Normandie Niémen, 13397 Marseille cedex 20, France. E-mail: s.viel@univ-cezanne.fr; Fax: +33 (0)4 9128 2897; Tel: +33 (0)4 9128 8900

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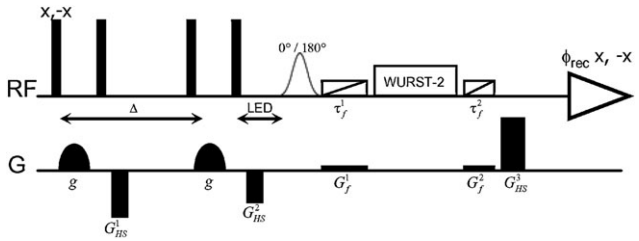

Fig. 1 Pulse sequence of the 3D Hadamard-encoded DOSY-TOCSY experiment (unless specified, all pulses have phase *x*).

In this communication, we illustrate the potential of this methodology and propose a pulse sequence based on Hadamard encoding that yields, under favourable circumstances, the TOCSY spectra of each mixture component in a matter of minutes, thereby affording a large reduction in experimental time with respect to currently available experiments. Because TOCSY is traditionally regarded as a tool of choice for mixture analysis[24] and offers, with respect to COSY, enhanced spectral resolution due to in-phase off-diagonal cross-peaks, this preliminary report focuses on the DOSY-TOCSY experiment. A few groups have already combined diffusion encoding with TOCSY (DETOCSY, PFG-TOCSY, DO-TOCSY, and DECODES).[5] While 3D displays were not systematically synthesized, three of these implementations could (directly or indirectly) reveal the TOCSY spectrum of the individual mixture components. However, the considerable time requirements of the corresponding pulse sequences (several hours) make them totally unsuitable for routine analysis. Fig. 1 shows the 3D Hadamard-encoded DOSY-TOCSY pulse sequence‡ proposed in this work, where the diffusion encoding period precedes the Hadamard-encoded TOCSY block in agreement with the nomenclature clarified by Nilsson *et al.*[9] This encoding is achieved by monopolar gradients, and the sequence incorporates a *z* filter delay (LED)[5] and a homospoil gradient, to allow eddy currents to dissipate and residual transverse magnetization to dephase, respectively. Next, the Gaussian polychromatic pulse is applied, according to the on/off (0°/180°) excitation scheme advocated by Freeman and coworkers,[22] and this is followed by an 80 ms isotropic mixing period employing WURST-2 adiabatic pulses used for reducing RF field inhomogeneity artefacts.[25] In order to improve spectral quality and reduce phase cycling, single-scan ZQC filters were applied before and after the mixing period.[26] Finally, a strong spoiler gradient was applied prior to signal acquisition to remove any residual transverse magnetization. All NMR experiments were carried out at 300 K on a BRUKER AVANCE 500 MHz spectrometer fitted with a 5 mm triple broad band inverse $^{1}H/^{31}P/X$ BRUKER probe equipped with a 56 G cm^{-1} gradient coil. A simple mixture was first adopted for this proof of principle (Fig. 2). The irradiation frequencies of the 90 ms phase-encoded Gaussian pulses used for Hadamard encoding were selected automatically by running a peak picking on a ^{1}H spectrum processed with a 10-Hz line broadening. Ten irradiation frequencies were obtained, while a 16 × 16 Hadamard matrix was chosen due to computer constraints. Fifteen gradient values from 9.0 to 33.9 G cm^{-1} in equal steps of gradient squared were used. A simple two-step phase cycle was employed, giving a total experimental time of ~20 min.†

In Fig. 2, the TOCSY spectrum of the mixture acquired with a conventional 2D TOCSY experiment based on the pulse sequence by Thrippleton and Keeler[26] is compared to the sub TOCSY spectra achieved with the pulse sequence in Fig. 1, showing that all expected correlations can be clearly detected. In most 3D DOSY experiments published so far, the minimum experimental time was in the range of several hours, especially when many gradient pulses were used. This is to be compared to the pulse sequence reported in Fig. 1, which gave satisfactory sub TOCSY spectra in a few minutes. Furthermore, to preliminarily evaluate the impact of signal overlapping on the outcome of the proposed 3D Hadamard-encoded DOSY-TOCSY experiment, a two-component mixture (propanol and 2-butanol), whose ^{1}H spectrum shows complete overlapping of the terminal CH_3 resonances (at 0.9 ppm), was analyzed. Fig. 3 compares the 2D Hadamard-encoded

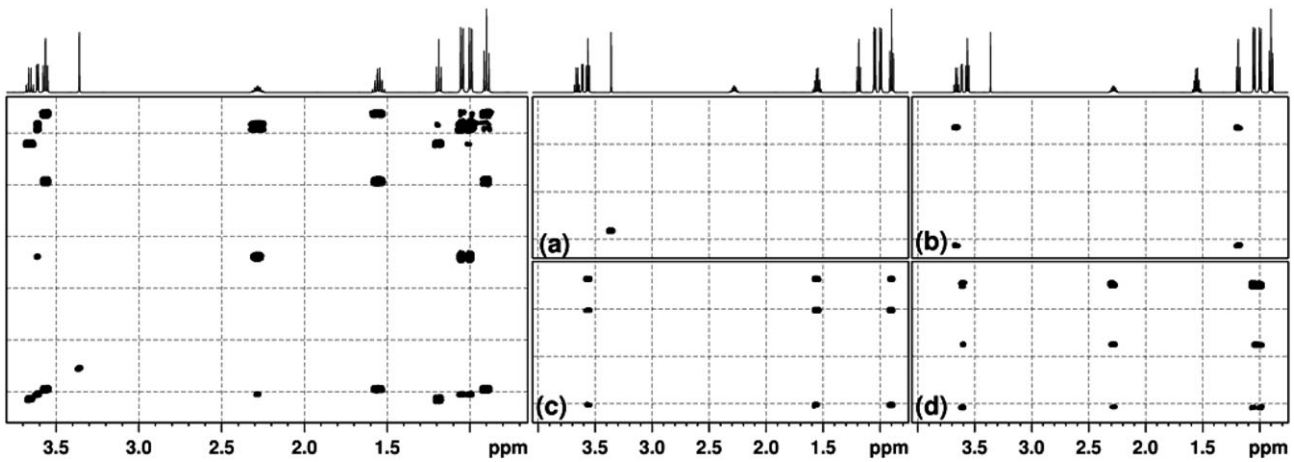

Fig. 2 [Left] TOCSY spectrum of a mixture of methanol, ethanol, propanol, and valine. [Right] Sub TOCSY spectra from the 3D Hadamard-encoded DOSY-TOCSY experiment on the same mixture at (a) 1.32, (b) 1.05, (c) 0.89, and (d) 0.63 × 10^{-9} m^2 s^{-1}, corresponding to methanol, ethanol, propanol, and valine, respectively. These *D* values equalled within ±2% those determined by conventional DOSY experiments. The 1D ^{1}H spectrum is shown at the top.

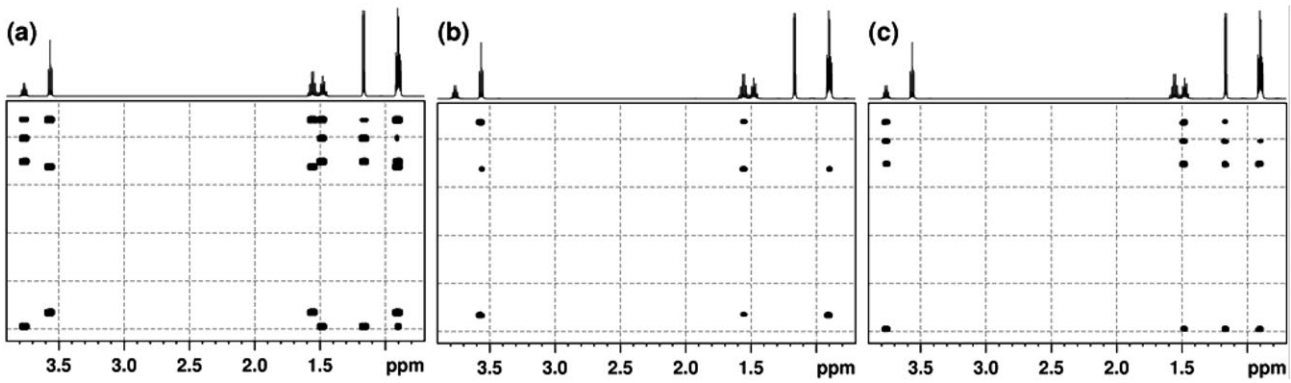

Fig. 3 (a) 2D Hadamard-encoded TOCSY spectrum of a mixture of propanol and 2-butanol. (b and c) Planes from the 3D Hadamard-encoded DOSY-TOCSY experiment recorded on the same mixture at (b) 9.5 and (c) 8.1×10^{-10} m^2 s^{-1}, corresponding to propanol and 2-butanol, respectively. These D values equalled within ±2% those determined by conventional DOSY experiments. The 1D ^{1}H spectrum is shown at the top.

TOCSY spectrum of this mixture to the TOCSY planes obtained for each substance with the pulse sequence of Fig. 1 (total experimental time ~13 min). As can be seen, each diffusion-encoded TOCSY plane exhibits all the expected cross-peaks of the respective mixture component, showing that the cluster of peaks at 0.9 ppm has been totally resolved. Similarly to Nilsson *et al.*,[16] the only absent peak is the diagonal (0.9; 0.9) ppm peak, whose weighted-average diffusion coefficient falls outside the diffusion ranges of the mixture components.

Importantly, in Hadamard NMR spectroscopy, the spectral resolution in the indirect dimension is approximately inversely proportional to the length of the selective excitation or inversion pulse. Steinbeck and Chmelka estimated the time saving accessible by Hadamard encoding,[23] and showed that it is inversely proportional to the Hadamard matrix dimension, which relates to the number of irradiated sites. Clearly, as the number of excited frequencies increases, this time saving will be scaled down correspondingly. In addition, with respect to currently available experiments such as COSY-IDOSY and DQFCOSY-IDOSY, the 3D Hadamard-encoded DOSY-TOCSY experiment outlined in this work bears two sensitivity penalties because more RF pulses are used (including the long, selective ones) and the sequence is built on the stimulated echo, which is intrinsically less sensitive than the Hahn echo (although this may be somewhat compensated for by the predominance of T_1 *vs.* T_2 relaxation). However, it has the advantage of yielding in-phase cross-peaks. Data are hence processed in the phase sensitive mode, which represents a substantial resolution enhancement compared to the above mentioned sequences, which require magnitude mode processing to compensate for the phase evolution that occurs during the spin echo. Overall, our pulse sequence is mostly adapted to the study of small molecules, as the rather long excitation pulses used for Hadamard encoding make it probably unsuitable for the investigation of large molecular weight species. Finally, while this preliminary report strictly focuses on the 3D DOSY-TOCSY experiment, the outlined methodology could in principle be applied to other 3D homonuclear (NOESY, COSY) experiments. Investigations along these lines are currently in progress in order to analyse complex mixtures.

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‡ Note that, although some of the features in Fig. 1 can be found in the STEP-DOSY experiment by Bradley *et al.*,[27] these authors did not intend to generate diffusion-encoded TOCSY spectra.

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Improved 3D DOSY-TOCSY experiment for mixture analysis

Stéphane Viel* and Stefano Caldarelli

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This section lists all relevant experimental parameters and describes the setup that was used to obtain the results described in the article. The focus is only on the 3D Hadamard-encoded DOSY-TOCSY experiment, since the parameters of all other experiments (conventional and Hadamard-encoded 2D TOCSY experiments) have been described elsewhere (see in the article references 26 and 22, respectively).

1. Samples

The first mixture was an equimolar mixture of methanol, ethanol, propanol, and valine at 0.1 M in D_2O, whereas the second was an equimolar mixture of propanol and 2-butanol at 0.1 M in D_2O.

2. Acquisition parameters

General. *First Mixture.* 8192 complex data points were acquired with a spectral width of 2125.85 Hz, giving a total acquisition time of 1.92 s. A relaxation delay of 0.5 ms was chosen. The 90° pulse was 9 µs. The diffusion gradient pulse duration was 2.0 ms and the diffusion delay Δ was 200 ms. The diffusion gradients were sine shaped. All other gradients were rectangular. The duration of the first two homospoil gradient pulses was 1.0 ms whereas the third was 3.0 ms, and their amplitudes were -32.0, -17.4, and 28.7 G/cm, respectively. The gradient recovery delay was set to 0.15 ms. The LED was 5 ms and the delay between the last homospoil gradient and the last RF pulse was 2 ms. For the ZQC filters, the swept-frequency pulses were adiabatic 180° CHIRP pulse (90 kHz sweep rate and 10% truncation). τ_f^1 and τ_f^2 were equal to 30 and 18 ms, resp., and their RF power were 2.9 and 3.8 kHz, resp. These pulses were applied in the presence of a rectangular gradient (2.2 and 2.5 G/cm, resp.). Homonuclear mixing was achieved with frequency-swept adiabatic 180° WURST-2 pulses (16 kHz sweep rate and 10% truncation), exhibiting a duration of 0.25 ms and a RF power of 7.3 kHz. These pulses were embedded in a MLEV-16 supercycle, and this cycle was repeated to give a total mixing time of 80 ms. *Second Mixture.* 4096 complex data points were acquired with a spectral width of 2062.71 Hz, giving a total acquisition time of 0.99 s. A relaxation delay of 2.0 ms was chosen. The 90° pulse was 8.45 µs. All other parameters were as above.

Diffusion encoding. Diffusion encoding was performed using monopolar gradients, as opposed to bipolar gradients usually employed in DOSY experiments to minimize spectral distortions due to eddy currents.[1] This solution was chosen in order to reduce the number of scans and maximize the experimental speed, since the use of bipolar gradients implies extra RF pulses which, in addition to reducing the sensitivity, impose additional phase cycling to prevent the acquisition of spurious signals. With the refinement of active gradient shielding in modern NMR probes and the generalisation of gradient-pulse shaping, eddy current suppression is not necessarily of major concern, especially when it comes to the analysis of mixtures of small molecules for which relatively low intensity gradient pulses are required. An additional advantage of bipolar gradients relies on the inversion RF pulses placed in the middle of the diffusion coding and decoding periods, which allow chemical shift evolution to be refocused, hence preventing the formation of zero quantum coherences (ZQC).[2] These pulses also compensate for background gradients,[3] an issue that may jeopardize the accuracy of the measured *D* values, but which is less relevant here because the primary objective is to separate the mixture components rather than strive for optimal accuracy.

Phase cycling. A simple two-step phase cycle was employed, alternating the phase (*x*, -*x*) of the first pulse only, in order to minimize the effects due to relaxation during the pulse sequence. Most probably, substantially cleaner results could have been obtained through the use of adequate, more extensive phase cycling. However, to maximize experimental speed, the smallest number of scans required to achieve satisfactory results was chosen. Moreover, to avoid accidental magnetization refocusing, all homospoil gradients, including those used for the ZQC filters, should ideally[4,5] be orthogonal.

Hadamard. As described in the article, for the first mixture, 10 irradiation frequencies were selected from the peak picking routine. In Hadamard NMR spectroscopy, this implies to use a Hadamard matrix of dimension $N = 2^m$, where *m* is an integer such that 2^m is equal to or higher than 10. The corresponding *m* value is 4 ($N = 16$).‡ The matrix dimension gives the number of polychromatic selective pulses that must be used, and hence 16 Gaussian 90-ms phase-encoded selective pulses were used here. These pulses were automatically created by a macro provided by BRUKER. For the second mixture, the same procedure applied but 6 irradiation frequencies were found, requiring $N = 8$.

3. Experimental setup

Acquisition. The 3D cube showed ^{1}H chemical shifts in the F1 and F3 dimensions (F3 being the directly observed dimension) and gradient amplitudes in F2. To obtain this cube, sixteen 2D experiments were sequentially recorded (one for each Hadamard-encoded Gaussian pulse). For each 2D experiment, the amplitude of the diffusion gradient *g* was varied from 2.1 to 33.9 G/cm.[†] This procedure was chosen because it was the simplest to implement with respect to the acquisition software in use (XwinNMR 3.5). Each FID of these 2D experiments was composed of 2 transients, and 4 dummy scans were run before the first 2D experiment only. Therefore, the total experimental time for the first mixture was:

$$\left(N \times N_{grad} \times 2 + 4\right) \times \left(1.92 + 0.5\right) \approx 20\,\text{min}.$$

Similarly, for the second mixture, the total experimental time was:

$$\left(N \times N_{grad} \times 2 + 4\right) \times \left(0.99 + 2.0\right) \approx 13\,\text{min}.$$

Processing. Data processing sequentially required the use of XwinNMR 3.5 and Topspin 2.0 from BRUKER.

[XwinNMR 3.5] A series of macros (AU programs) were written to process the so-obtained 2D files and decode the Hadamard-encoded data. This preliminary processing yielded 16 diffusion-encoded two-dimensional planes (with 1024x1024 data points), one plane for each of the 16 experimentally used gradient amplitudes.[†] The cross-peaks shown in these 2D spectra were obtained through a symmetrisation procedure similar to that described in reference 22.

[Topsin 2.0] All the above mentioned diffusion-encoded planes (except the first one) were written as F3-F1 planes in a 3D file exhibiting 1024, 128, and 1024 points in F1, F2, and F3, resp. In other words, along the F2 dimension, all planes were blank except the first 15. Finally, the diffusion dimension was processed by using the implemented *dosy3d* command. The F3-F1planes shown in Fig. 2 and Fig. 3 were then extracted from this cube by scanning the F2 dimension.

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Notes

[‡]Strictly speaking, a Hadamard matrix of dimension $N = 12$ could have been used as well, but our experimental setup required 16 (due to programming reasons only).

[†]Importantly, when processing the data of both mixtures and looking at the exponential signal decays, the first point was systematically an outlier. It was thus removed before processing the whole 3D cube. This explains why, in the text, only 15 gradient amplitudes are specified, although 16 were used in total. However, to properly compare our results, the total experimental time mentioned in the text was calculated by accounting for all 16 gradient amplitudes.

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