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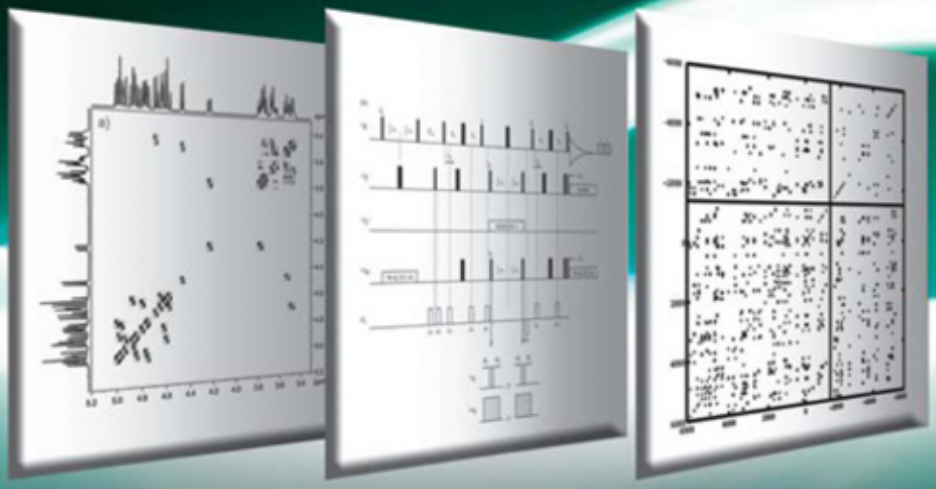

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Multidimensional NMR Methods for the Solution State

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Multidimensional NMR Methods for the Solution State

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Series Preface

The *Encyclopedia of Nuclear Magnetic Resonance* was published in eight volumes in 1996, in part to celebrate the fiftieth anniversary of the first publications in NMR in January 1946. Volume 1 contained an historical overview and ca. 200 short personal articles by prominent NMR practitioners, while the remaining seven volumes comprise ca. 500 articles on a wide variety of topics in NMR (including MRI). Two "spin-off" volumes incorporating the articles on MRI and MRS (together with some new ones) were published in 2000 and a ninth volume was brought out in 2002. In 2006, the decision was taken to publish all the articles electronically (i.e. on the World Wide Web) and this was carried out in 2007. Since then, new articles have been placed on the web every three months and a number of the original articles have been updated. This process is continuing. The overall title has been changed to the *Encyclopedia of Magnetic Resonance* to allow for future articles on EPR and to accommodate the sensitivities of medical applications.

The existence of this large number of articles, written by experts in various fields, is enabling a new concept to be implemented, namely the publication of a series of printed handbooks on specific areas of NMR and MRI. The chapters of each of these handbooks will comprise a carefully chosen selection of Encyclopedia articles relevant to the area in question. In consultation with the Editorial Board, the handbooks are coherently planned in advance by specially selected editors. New articles are written and existing articles are updated to give appropriate complete coverage of the total area. The handbooks are intended to be of value and interest to research students, postdoctoral fellows, and other researchers learning about the topic in question and undertaking relevant experiments, whether in academia or industry.

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November 2009

Volume Preface

Over ten years passed between the first recognition of the potential of NMR methods based on Fourier transformation of the response to a radiofrequency pulse and the practical realization of that potential, by Ernst and Anderson, in 1966. The effect on the practice of NMR was rapid and profound, with pulse-Fourier transform equipment quickly supplanting continuous wave spectrometers. The great improvement in sensitivity achieved by this method opened up areas of the periodic table that had until then been largely unexplored by NMR, and the chemical application of multiple pulse experiments such as inversion recovery and the spin echo began in earnest.

It was only five years later, in 1971, that Jean Jeener proposed another technique, two-dimensional or 2D NMR spectroscopy, that was to have equally far-reaching implications. This time it took just four years for the first successful experiments to be reported, again by Richard Ernst and his colleagues, and once again the new methods were rapidly and widely adopted. Multidimensional NMR methods have since transformed the way NMR is used in chemistry, biology, physics, and medicine, to the extent that they are now part of the routine vocabulary of chemistry and of structural biology.

One of the most engaging features of NMR is its continuing ability to surprise. Despite over half a century of intensive study of the phenomenon of magnetic resonance, new discoveries and new developments in technique are still being made, and the flow of new ideas continues unabated. One of the most fruitful areas of development in recent years has been in methods for speeding up 2D and high-dimensionality experiments. Thus it is now possible in some cases to acquire a complete 2D spectrum in a few seconds, or to acquire data correlating five or six spectral dimensions overnight, with time savings of several orders of magnitude. Thus while this handbook contains authoritative accounts of techniques such as COSY, NOESY, and TOCSY that have acquired the status of classics, it also includes a range of articles on techniques that have been developed within the last few years, each written by the leader of the relevant field.

This handbook is structured in four parts. The first opens with a historical introduction to, and a brief account of, the practicalities and applications of multidimensional NMR methods, followed by a definitive survey of their conceptual basis and a series of articles setting out the generic principles of methods for acquiring and processing multidimensional NMR data. In the second part, the main families of multidimensional techniques, arranged in approximate order of increasing complexity, are described in detail, from simple J-resolved spectroscopy through to the powerful heteronuclear 3D and 4D methods that now dominate the study of structural biology in solution. The third part offers an illustrative selection from the very wide range of applications of multidimensional NMR methods, including some of the most recent developments in protein NMR. Finally, the fourth part introduces the idea of multidimensional spectra containing nonfrequency dimensions, in which properties such as diffusion and relaxation are correlated.

The literature of multidimensional NMR began with three papers in 1975, then nine in 1976, and fifteen in 1977, and now contains many tens of thousands of papers. Any attempt to survey the field must therefore necessarily be very selective, not to say partial. In assembling this handbook, and the *Encyclopedia of Magnetic Resonance* with which its component articles are shared, we have

sought to provide both the new researcher and the established scientist with a solid foundation for the understanding of multidimensional NMR, a representative if inevitably limited survey of its applications, and an authoritative account of the latest progress in the development of multidimensional techniques.

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April 2010

PART A
Principles

Chapter 1
Multidimensional NMR: an Introduction

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1.1 INTRODUCTION

The first demonstration of pulse Fourier transform NMR spectroscopy brought a great improvement in the sensitivity of NMR,[1] and a corresponding widening of its range of applications. Although it was far from obvious at the time, the introduction of FT methods had another, even more profound, consequence for the scope and power of NMR spectroscopy. The change from experiments in which NMR signals were excited and measured simultaneously, as in continuous wave (CW) NMR, to pulsed methods, in which excitation and detection are separated in time, gave the experimenter freedom to manipulate the chemical or physical information content of the data measured, and initiated a florid growth in experimental NMR techniques that has lasted 40 years and shows no sign of abating.

The biggest breakthrough enabled by the separation of excitation and detection in pulsed Fourier transform NMR was the development of multidimensional NMR, in which the idea of a spectrum as a record of signal strength as a function of frequency, $S(F)$, was extended into multiple frequency dimensions, $S(F_1, F_2, \ldots)$. Instead of simply acquiring a free induction decay $s(t)$ following radiofrequency (RF) excitation, free induction decays $s(t_n)$ are acquired using a pulse sequence containing one or more evolution times $t_1, t_2, \ldots t_{n-1}$ for a series of equally spaced values of the evolution times. The complete data matrix $s(t_1, t_2, \ldots t_{n-1}, t_n)$ is then Fourier transformed with respect to $t_1, t_2, \ldots t_{n-1}$, and t_n to yield an n-dimensional spectrum $S(F_1, F_2, \ldots F_{n-1}, F_n)$. (Although the term "multidimensional spectroscopy" in NMR spectroscopy encompasses a wide range of techniques, multidimensional Fourier transform methods dominate, and the term is used here in this sense unless specifically indicated otherwise). By far the commonest family of techniques is two-dimensional, or 2D, NMR spectroscopy, in which free induction decays $s(t_2)$ are acquired for NI increments of an evolution time t_1, and the resultant time-domain data matrix $s(t_1, t_2)$ is Fourier transformed first with respect to t_2 and then with respect to t_1 to give the 2D spectrum $S(F_1, F_2)$. The free induction decays

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are sampled every $\Delta t_2 = 1/SW2$ seconds and the evolution time is incremented in steps $\Delta t_1 = 1/SW1$, giving a 2D spectrum with a spectral width of $SW1$ (in hertz) in the F_1 direction and $SW2$ in F_2.

The first advantage of such multidimensional methods is the potential for improved signal resolution. For a normal (or one-dimensional, 1D) spectrum with a spectral width SW, if the frequency range covered by a typical peak is W, then the maximum number of peaks that could, in principle, be resolved is of the order of SW/W. For a 2D spectrum the corresponding number is $SW1 \times SW2/W^2$, which, for typical spectra, represents a large improvement over the 1D case. The second advantage of multidimensional methods is that both the way in which signals are dispersed in a given frequency dimension, and the relationships between those dimensions, depend on the pulse sequence used and are under the direct control of the experimenter. Without this ability to disperse signals differently in different dimensions, there would be no resolution advantage—signals would simply be spread along the diagonal $F_1 = F_2$, with no better resolution than in a 1D spectrum. It is possible to control signal behavior by manipulating the sequence of RF pulses used because even under routine experimental conditions the response of an NMR spin system to RF excitation is nonlinear. NMR is almost unique in this respect, other spectroscopic methods requiring special hardware such as high-powered lasers or microwave amplifiers to drive the spectroscopic response out of the linear regime.

Although the archetypal multidimensional NMR experiments use multiple Fourier transformation of data measured with multiple time domains, neither the Fourier processing nor the measurement of time-sampled data is essential for obtaining the advantages of resolution and enhanced information content. Thus the Fourier transform can be replaced with alternative algorithms (see Chapters 4–10). Such methods are particularly useful where data are sampled nonuniformly in one or more of the time domains, and for high dimensionality ($n>3$) experiments. Similarly, it is also possible to generate multidimensional spectra from data acquired not as a function of evolution time(s), but of experimental variables such as pulsed field gradient strength, as in diffusion-ordered spectroscopy (see Chapter 36), or from time-sampled data where the signal behavior is not oscillatory and hence Fourier processing is inappropriate (see Chapter 37).

1.2 HISTORICAL BACKGROUND

Since the earliest experiments on NMR of liquids, there has been a constant battle to improve spectral resolution. At first, attention was concentrated on minimizing the contribution of static magnetic field inhomogeneity to signal linewidths; it then shifted to increasing the field strength. The introduction of superconducting magnets brought an immediate factor of two improvement in signal separation, but a much larger gain came in 1975 with the first publication of a two-dimensional NMR spectrum.[2] While the use of double-resonance experiments to unravel overlapping multiplets was by that stage commonplace, the idea of using double Fourier transformation of data acquired with two time dimensions had taken a relatively long time to reach fruition. It was first presented formally by Jean Jeener at a conference in Baško Polje, in what is now Croatia, in 1971, but initial experiments were defeated[3] by what is now termed t_1-*noise*.[4] Jeener's experiment consisted simply of two RF pulses separated by a variable evolution time t_1. This pulse sequence is now known as COSY (Correlated SpectroscopY), because it correlates pairs of signals that share a scalar coupling, and over 30 years later it is still one of the most widely used of multidimensional NMR techniques (see Chapters 2, 12 and 13). A paper describing the principles of the new experiment was drafted in November 1971 but never submitted, although the results later appeared in the doctoral thesis of Gerrit Alewaeters ("Een Twee Impulstechniek in Kernmagnetische Resonantie in Vloeistoffen", Vrije Universiteit Brussel, 1976). (The unpublished preprint is notable not just for its clear-sighted analysis of the potential of 2D NMR, but for listing among its advantages "very simple theory"!)

The first successful experiments using a second Fourier transformation were reported in April 1975 by the groups of the future Nobel laureate Richard Ernst,[5] using liquid-state COSY,[2] and of John Waugh,[6] using oscillations in solid-state cross-polarization to resolve heteronuclear dipolar couplings.[7] Later that year, Ernst reported the first liquid-state ^{13}C results, correlating proton-decoupled and proton-coupled ^{13}C spectra,[8] and, in January 1976, the group of Endel Lippmaa reported the use of a second Fourier transformation to record the bandshapes of individual ^{13}C resonances in solids.[9] These early experiments attracted immediate attention, establishing the principle that large gains

in signal resolution could be achieved by dispersing peaks in two independent dimensions F_1 and F_2, using double Fourier transformation of experimental data acquired using a pulse sequence with a variable evolution time t_1. Signal dispersion as a function of F_2 is the same as that in the normal spectrum, but dispersion in the indirect dimension F_1 is determined by the average signal evolution frequency during t_1, giving the experimenter control over the chemical information encoded in the indirect dimension.

In March 1976, the principles of two-dimensional NMR spectroscopy were laid out, and the directions of many future developments defined, in the classic paper of Aue *et al*.[10] This analyzed in detail the effects of the archetypal 2D NMR pulse sequence suggested by Jeener, which consists simply of two RF pulses separated by a variable evolution time t_1, and established a basic structure for a 2D pulse sequence: a preparation period, an evolution period t_1, and a detection period t_2. Amongst other things, this paper analyzed the COSY experiment for AX, AB, and larger spin systems, introduced the terms *cross peak* and *dia (diagonal) peak*, described the use of 2D Fourier methods for magnetic resonance imaging, analyzed the effect of static field inhomogeneity on 2D lineshapes, and illustrated the use of 2D NMR to detect zero- and double-quantum coherences. In a COSY experiment, diagonal peaks appear close to the line $F_1 = F_2$ and arise from signals that remained at the same chemical shift after the second pulse, and cross peaks appear away from the diagonal and arise where scalar coupling causes signals to change chemical shift between t_1 and t_2. A COSY spectrum thus anatomizes the scalar coupling relationships in a proton spectrum, cross peaks appearing wherever a J-coupling is detected between two protons.

At that time spectrometer computers had very limited memory, typically around 16 kilobytes to store both the spectrometer control program and the NMR data acquired, so there was a strong incentive to investigate techniques that, unlike COSY, yield only a narrow range of frequencies in F_1. The next developments were therefore in J-resolved (or 2D J-) spectroscopy (see Chapter 11), in which the evolution period t_1 consists of a modulated spin echo. Here the indirect dimension displays only multiplet structure, and hence requires a much smaller spectral width than the normal spectral dimension F_2. Ernst's group demonstrated a homonuclear J-resolved experiment[11] (giving as a by-product a method for measuring proton spectra without multiplet structure, a prize that had been sought for many years), and that of Freeman developed several variants of heteronuclear 2D J-spectroscopy.[12,13] The latter experiments showed the benefits of suppressing field inhomogeneity contributions to linewidths in the indirect dimension, a gain anticipated in the earlier one-dimensional J-spectroscopy method of Freeman and Hill.[14,15]

It was recognized from the outset that there were problems displaying the results of 2D experiments, because of the nature of the 2D lineshapes, and, as a result, almost all early results displayed the modulus, or absolute value, of the signal rather than its real or imaginary part. The problem of the "phase-twist" lineshape was analyzed by Bodenhausen *et al*.,[16] in a paper that also described and analyzed some of the signal artifacts seen in heteronuclear J-resolved spectra, and by Bachmann *et al*.[17] Both the phase-twist and the artifact analyses were to have lasting impact, the former in the development of methods for phase-sensitive display of 2D spectra with pure absorption mode peaks, and the latter as the stimulus for the development of EXORCYCLE,[18] the first phase cycle to allow the selection of a desired coherence transfer pathway and the prototype for hundreds of subsequent phase cycles.

It was clear from the outset that one of the key applications of 2D NMR would, when instrumentation and software permitted, be to the study of biomolecules. As early as 1977, a homonuclear J-resolved spectrum was reported for a mixture of amino acids,[19] rapidly followed by a J-resolved spectrum of the protein bovine pancreatic trypsin inhibitor.[20,21] This was the beginning of an area of research that was to lead to the award of a second Nobel prize involving multidimensional NMR, to Kurt Wüthrich,[22] and to the establishment of NMR as the method of choice for the determination of 3D structures of proteins and other biopolymers in solution[23] and as one of the primary tools of structural biology.

Up to this point, the highest resolution 2D technique studied was COSY, generating spectra in which signals were dispersed according to the proton chemical shift in both frequency dimensions. The next major step, in 1977, was the extension to heteronuclear 2D correlation (see Chapter 22), in which signals are dispersed as a function of proton chemical shift in one frequency dimension and carbon in the other,[24] offering almost an order of magnitude improvement in resolution because of the combined effects of the wider ^{13}C chemical shift range and the narrower ^{13}C peaks. It was quickly realized[25] that experiments recording

proton free induction decays ("indirect detection") should in principle offer much better sensitivity than those recording ^{13}C signals ("direct detection"). The same paper also made explicit the idea, left implicit in earlier work, of a mixing period between t_1 and t_2. The archetypal structure of a 2D NMR pulse sequence was thus established as consisting of preparation, evolution, mixing (also known as *transfer*—see Chapter 2) and detection periods.

Initially, direct detection methods for heteronuclear chemical shift correlation prevailed, with practical experiments using ^{13}C detection[26] because of the difficulty of achieving adequate suppression of the signals of protons not coupled to ^{13}C. As instrument stability improved, indirect detection methods such as HMQC[27] and HSQC[28] for correlation through one-bond couplings, and HMBC[29] for long-range correlation, took over. The directly detected experiment did however lead, via the loss of its evolution period, to the 1D INEPT pulse sequence, which is now a ubiquitous building block in 2D and 3D pulse sequences for biomolecular structure determination. Indirect detection pulse sequences were also subsequently developed for more specialized purposes, for example, the measurement of long-range heteronuclear coupling constants (see Chapter 23).

As the field of 2D NMR began to consolidate, a number of papers were published that examined the technical underpinning of the method. An analysis of signal-to-noise ratio in 2D techniques[30] showed that the sensitivity penalty on moving from one dimension to two was much smaller than had generally been supposed. Analytical and computational results for the 2D spectra of strongly coupled spin systems[31–34] allowed both direct and iterative analysis of 2D spectra for the extraction of accurate spin system parameters. The application of the projection–cross-section theorem to 2D NMR[35] showed, amongst other things, why it was not possible to obtain an absorption mode decoupled spectrum by 45° projection of a homonuclear J-resolved spectrum; ways around this limitation were only found much later (see Chapter 11).

The steadily increasing number of applications of 2D NMR methods to chemical and biochemical problems brought further stimulus to technical development, for example, the application of heteronuclear correlation methods to the $^{1}H-^{31}P$ spin pair[36] and the investigation of $^{13}C-^{1}H$ dipolar couplings by application of the separated local field (SLF) experiment to a liquid crystalline sample[37] (see Chapter 31). The next big step, however, was the introduction in 1979 of what is now known as the NOESY (*Nuclear Overhauser Effect Spectroscopy*) experiment[38,39] (see Chapter 18), which correlates signals through the exchange of longitudinal magnetization. Although, as the name suggests, it is most often used for the detection of nuclear Overhauser effects, which are caused by through-space dipolar interactions, the same pulse sequence may be used to detect the transfer of magnetization through chemical exchange (where it is sometimes referred to as the *EXSY* (EXchange SpectroscopY) sequence; see Chapter 21. The NOESY experiment and its derivatives were to play a crucial role in the development of NMR as a tool for structural biology,[23,40] allowing, for the first time, the efficient measurement of proton–proton distances in macromolecules. It was also at this stage that the first reviews on 2D methods began to appear.[41,42]

The COSY experiment is very effective at identifying coupling relationships between spins, but ambiguities frequently arise where resonances overlap. Thus the observation of cross peaks at chemical shifts (δ_1, δ_2) and (δ_2, δ_3) could mean that there is a chain of three protons H_1, H_2 and H_3 with couplings J_{H1H2} and J_{H2H3}, or it could simply be that there are two unrelated protons H_{2a} and H_{2b} at the same chemical shift δ_2 with couplings J_{H1H2a} and J_{H2bH3}, and the two spin systems H_1H_{2a} and $H_{2b}H_3$ are completely unrelated. Such ambiguities can be resolved by adding an extra coherence transfer stage to COSY, giving the RELAY (relayed correlation spectroscopy) pulse sequence, which was first described in 1982.[43] At around the same time, the analogous experiment for heteronuclear correlation, in which protons coupled to protons coupled to phosphorus were identified, was also described[44] (see Chapter 15).

The use of 2D NMR to probe multiple-quantum coherences (see Chapters 17 and 32) dates back to Ernst's classic 1976 paper,[10] but chemical applications began in earnest with the extension of the INADEQUATE experiment,[45] in which phase cycling of a pulse pair at the end of a modulated spin echo is used to filter out all ^{13}C signals from molecules with only one ^{13}C spin, to two dimensions.[46] By correlating the signals of directly bonded carbons, this allowed the carbon skeleton of a molecule to be traced out bond by bond, albeit with very low sensitivity. The process of filtering signals through multiple-quantum coherence was applied in more general fashion in multiple-quantum filtered (MQF) COSY.[47] Double-quantum filtration suppresses signals from protons with no couplings, while higher

order filters suppress progressively more and more signals, simplifying spectra.

Filtration experiments such as INADEQUATE and MQF-COSY are designed to improve resolution by *reducing* the number of signals in a 2D spectrum. A further class of homonuclear 2D correlation experiments that complemented COSY and NOESY, and supplanted RELAY, was introduced with the TOCSY (TOtal Correlation SpectroscopY)[48] experiment, also known as HOHAHA (HOmonuclear HArtmann HAhn), in 1983 (see Chapter 16). TOCSY sets out to *increase* the information content of a 2D spectrum by correlating all the spins in a scalar coupling network, using a pulse sequence containing a spin lock period that allows the sequential transfer of magnetization through couplings. Thus while cross peaks arise in COSY where two spins are coupled, and in NOESY where they are close in space or are undergoing mutual chemical exchange, in TOCSY cross peaks can appear for all pairs of protons that are connected by a continuous chain of scalar couplings. TOCSY is thus particularly useful in protein NMR, where each amino acid represents a single isolated network of coupled spins, and different classes of amino acids give different characteristic patterns of TOCSY cross peaks.

Shortly after the introduction of TOCSY, a second class of 2D pulse sequence appeared that also used spin locking, but for a different purpose. The ROESY experiment,[49] as it became known (see Chapter 19), was designed to circumvent the problem that the magnitude of the nuclear Overhauser effect passes through zero as the molecular rotational correlation time approaches the Larmor frequency, dividing the small-molecule regime (rapid motion, positive Overhauser effects) from the large (slow motion, negative Overhauser effects). In ROESY, the spin lock period allows spins to exchange transverse magnetization M_{xy} (as opposed to longitudinal magnetization M_z in NOESY). The effect is to make all species behave as small molecules do in NOESY, giving cross peaks with sign opposite to that of the diagonal peaks, independent of the timescale of molecular motion. Chemical exchange will also give rise to cross peaks, as in NOESY, but this time they are easily distinguished from ROE (rotating-frame Overhauser effect) cross peaks because they have opposite signs. ROESY is significantly more difficult both to perform and to interpret than NOESY, but is useful both for the structural and conformational analysis of intermediate-sized molecules and for studying larger molecules, where spin diffusion can cause problems in NOESY. The practical and interpretational difficulties with ROESY arise because the spin lock period allows other types of coherence transfer as well as the ROE, notably TOCSY-type transfer (see Chapter 20). It is therefore important both to design the spin lock irradiation to maximize discrimination between the ROE and competing transfer mechanisms, and to allow for the existence of the latter effects when analyzing ROESY spectra.

The great majority of 2D spectra produced in the early years used absolute value mode display of the spectral data, to avoid the complications of the phase-twist lineshape and the need to adjust zero and first-order phase corrections in both dimensions, although phase-sensitive mode was sometimes used when plotting cross sections through well-resolved spectra. There are clear disadvantages to using absolute value calculation: its nonlinearity means that signal intensities are distorted where peaks overlap, and severe weighting functions are needed to avoid peak shapes with very wide skirts, degrading both resolution and signal-to-noise ratio. There were therefore strong incentives to devise ways to generate signals with pure absorption mode lineshapes. The first two general solutions to the problem were the hypercomplex method of States *et al.*[50] and the TPPI (time-proportional phase incrementation) method of Marion and Wüthrich[51]; their relative merits have been assessed by Keeler and Neuhaus.[52] Both are still in use, as are hybrids of the two, while absolute value display is still often used for COSY and HMBC experiments. In the case of HMBC, this is because multiplets in F_2 are modulated by scalar couplings, while in COSY, the diagonal and cross peaks are 90° out of phase. (Another reason for favoring absolute value display in COSY experiments is that the antiphase character of the cross-peak multiplet structure can make the time-symmetric weighting functions used to generate acceptable lineshapes a close match to the time-domain signal envelope, approximating matched filtration and giving the absolute value COSY experiment surprisingly good sensitivity.) One major advantage of phase-sensitive homonuclear correlation experiments such as double-quantum filtered (DQF) COSY[53] and exclusive correlation spectroscopy (E.COSY)[54] (see Chapter 14) is that they can allow the accurate measurement of coupling constants.

A third solution to the problem of generating absorption mode 2D lineshapes, which is the commonest in current use, came somewhat later[55] when the availability of actively shielded gradient coils enabled the use of pulsed field gradients to generate complementary echo and antiecho 2D datasets, which can be combined to give pure absorption lineshapes.[56] From the earliest days of 2D NMR, it was appreciated that the key to obtaining clean, informative spectra is to restrict the signals seen to those that have a particular history, or more formally, to suppress all coherences that do not follow the desired coherence transfer pathway[57] during the pulse sequence. Initially, this selection was done exclusively by phase cycling,[58] permuting the phases of RF pulses and of the receiver on successive transients during time averaging, but in recent years, field gradient pulses have been used extensively for coherence transfer pathway selection. Phase cycling is essentially a difference method, canceling out the unwanted signals while retaining the wanted ones, so any instrumental instability will lead to small amounts of the unwanted signals surviving phase cycling. These appear in the 2D spectrum as t_1-noise, streaks of pseudorandom signal along the F_1 direction at the F_2 frequencies of strong signals. One advantage of pulsed field gradients is that they do not rely on subtraction to suppress unwanted signals, so t_1-noise contributions from unwanted pathways are effectively removed. This does not mean, as has sometimes been asserted, that spectra measured using pulsed field gradient methods are free of t_1-noise, but rather that only the wanted signals should contribute to the t_1-noise.

It was clear from the outset that the principle of 2D NMR could be extended to further dimensions, but the practical realization of experiments in three[59] and four[60] dimensions (see Chapter 24) had to wait until computer storage capacities had increased sufficiently to cope with the large quantities of data involved. The primary drive for such extensions came from structural studies on biomolecules, where the extra resolution was critical both for assignment and for the measurement of NOEs. The availability of expression systems for producing proteins with ^{13}C and/or ^{15}N labeling led[61] in the later 1980s to the rapid development of heteronuclear 3D and 4D correlation methods (see Chapters 25, 26, 33 and 34), which can allow the detailed assignment and analysis of the spectra of proteins containing hundreds of amino acids, and to the maturation of multidimensional NMR into one of the two most powerful techniques in modern structural biology (the other being X-ray crystallography).

The explosive growth of 2D NMR and its rapid adoption by chemists meant that the advantages of two-dimensional display of spectral data rapidly became familiar, and were therefore extended to correlation techniques not based on multiple Fourier transformation of time-domain datasets. The most widely used class of experiment in this category is diffusion-ordered spectroscopy or DOSY[62,63] (see Chapter 36), in which Johnson in 1992 took the idea of distinguishing between signals of different species by measuring their rates of diffusion[64] and adapted it for 2D display. In DOSY, pulsed field gradient echo spectra acquired for different gradient amplitudes are analyzed to extract information on the rates of diffusion associated with different signals, and the results presented in 2D or 3D form with signals dispersed according to diffusion coefficient in one dimension. Methods based on numerical approximations to the inverse Laplace transform are also gaining popularity, allowing parameters such as relaxation times and diffusion coefficients to be correlated in multidimensional spectra (see Chapter 37).

There is an important distinction to be drawn between DOSY and other data analysis techniques based on statistical modeling of experimental data, and conventional multidimensional NMR, in which time-domain data are subjected to multiple Fourier transformation. In the latter case, the linearity of the Fourier transform ensures that the frequency-domain spectrum is a faithful representation of the frequencies present in the time-domain data: peaks appear where they belong. In spectra obtained by statistical modeling, the frequencies, diffusion coefficients or other parameters modeled are subject both to statistical uncertainty and to systematic distortion: where peaks end up depends both on the accidents of noise, and on the positions and amplitudes of all the other signals in the spectrum. Thus if two signals in a COSY experiment have the same F_2 frequency and F_1 frequencies of 4.9 and 5.0 ppm, the 2D spectrum will show peaks at $F_1 = 4.9$ ppm and $F_1 = 5.0$ ppm. If two signals in a 2D DOSY experiment have the same F_2 frequency and diffusion coefficients of 4.9 and $5.0 \times 10^{-10}\,m^2\,s^{-1}$, the DOSY spectrum will show a single signal at around $4.95 \times 10^{-10}\,m^2\,s^{-1}$ in the diffusion domain—irrespective of whether monoexponential, biexponential, or more sophisticated fitting is used.