Forum for European
Structural Proteomics

Research Infrastructures in Structural Proteomics: Assessment of Requirements for Bio-NMR Infrastructures

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Abbreviations: NMR (nuclear magnetic resonance), RI (research infrastructure), NOE (nuclear Overhauser effect), RDC (residual dipolar coupling), PDB (protein data bank)

Executive summary

As we mark the first decade of the 21st century, NMR spectroscopy is a well-established method for the structure determination of macromolecules and currently contributes about 15% of the biomolecular structures deposited in the Protein Data Bank (PDB). The availability of adequate NMR Research Infrastructures, equipped with state-of-the-art instrumentation including the most innovative accessories, is strategic for maintaining European Structural Biology at the forefront of the field worldwide. In view of the on-going and planned high-throughput and structural/functional proteomics projects within Europe, the European Commission (EC) considered it timely to assess present and future needs of NMR Research Infrastructures for such activities *from the users' perspective*. This assessment was carried out within the context of a Specific Support Action (Forum for European Structural Proteomics, FESP) that addresses large-scale infrastructures within Structural Proteomics in general (NMR spectroscopy, electron microscopy, protein production, bioinformatics, synchrotrons). To this end, a questionnaire was sent to users of bio-NMR Research Infrastructures at the European and national levels as well as to scientists of NMR research groups throughout Europe, both in academia and in the pharmaceutical and biotech industries. The findings of the survey are summarized in this report. They provide information about the current status of biological research within Europe utilizing NMR infrastructures, and are intended to contribute to the formulation of policies concerning infrastructure requirements for Structural Genomics / Structural Proteomics (SG/SP) and for Structural Biology (SB) over the next 5-10 years.

On the basis of this survey and on discussions with a number of leading scientists in the field of NMR applied to biological macromolecules, FESP recommends the following actions to the European Commission.

Bio-NMR Research Infrastructures play an essential role in SG/SP

Bio-NMR Research Infrastructures, equipped with the most advanced and updated instrumentation, are critical for the success and competitiveness of European SG/SP and SB. These infrastructures have been and are being extensively used by structural and molecular biologists, as well as by biologists in general; these scientists are exploiting the versatility and multiple powerful capabilities of NMR spectroscopy in the characterization of biological molecules and functional processes. Access to these large-scale facilities has been supported by the European Commission since 1994, and this support has been absolutely vital for the success of European Structural Biology. FESP strongly recommends continued funding of access to bio-NMR facilities. These Research Infrastructures (formerly referred to as Large Scale Facilities) are of essential importance for facilitating internationally competitive biological research. Funding the access scheme for large Research Infrastructures requires both direct support for user travel (including sample shipment), and support for technical staff and running costs at the NMR facilities.

Number of bio-NMR Research Infrastructures

Currently, there are five bio-NMR Research Infrastructures (RIs) that provide Transnational Access (see Appendix 2) funded by the EC. The aim is to provide the highest magnetic fields and most updated instrumentation at these RIs, which then network with other smaller, regional facilities to accommodate all the needs of the user pool and to optimize access by deploying a wide network with a balanced geographical distribution. Taken as a whole, the existing RIs are adequate to meet the current needs of the bio-NMR community, both in terms of instrumentation time and in the types of equipment available. However, it is foreseen that the demand will significantly increase in the near future, as NMR can be used not only for structure determination, but also for a wide range of emerging applications, such as the characterization of weak, transient interactions between biological macromolecules, screening studies of active pharmaceutical compounds and of metabolic profiles. The demand for NMR time is also going to increase markedly as the availability of samples for membrane proteins increases, thus

increasing the needs for access to solid-state NMR. From this broad range of applications, the considerable potential of NMR to make contributions to studies in a Molecular Systems Biology context is clearly emerging. Furthermore, a new pool of users is to be expected as a result of the recent addition of new EU member states.

Procedures for the generation of automated NMR data acquisition routines should be enforced

There is a great need for automation in NMR data acquisition, handling and analysis that should be given a high priority in order to ramp up throughput. Efforts should be particularly directed toward data analysis, e.g. automatic assignment of resonances and NOE cross-peaks yielding structural restraints. Implementation of remote access is another priority, as this would ultimately reduce user expenses and time-consuming user travel to the NMR Research Infrastructures. This would require further investments in technical staff and protocols at the RIs but we believe that this would be very cost-effective overall.

NMR method development should be strengthened

Development and implementation of new technologies for macromolecular characterization and preliminary analysis by NMR in solutions and solids are essential for maintaining the competitiveness of European SG/SP and SB in both academia and industry, and require close collaborations between NMR manufacturers and the NMR community. Such developments include higher magnetic fields, improved sensitivity, further exploitation of cryogenic probe technology for solution and solid-state NMR, dynamic nuclear polarization (DNP), fast data acquisition, very high speed magic-angle spinning (MAS) and probes optimized for proton detection in solid-state NMR, and automation software and hardware. Methodological aspects to be addressed include NMR techniques to obtain structural restraints for high molecular weight systems (residual dipolar couplings, paramagnetic tags) and the combination of NMR with complementary techniques (e.g. X-ray crystallography, Small Angle X-ray Scattering, EM, etc.). These developments should be coordinated and supported at the European level, and FESP strongly recommends that the European Commission fund projects for attaining these objectives.

Background

European Bio-NMR Research Infrastructures have played a key role in the astonishing scientific breakthroughs that the Structural Biology community has witnessed during the last two decades. The impact of these large-scale research infrastructures on Structural Biology can be gauged by the increasing number of structures solved in solution through the use of NMR, currently numbering around 7000. The positive effect of NMR and Research Infrastructures is also increasing due to a growing awareness that the overall properties of biological macromolecules cannot be described only through individual snapshots. NMR has the ability to monitor and describe the range of conformations sampled by biomolecules in solution as well as their mobility over a wide range of time scales. This information is essential for fully describing the functional properties of biomolecules. Perhaps the most important future impact of NMR, however, will be in its ability to define the interfaces and kinetics of the myriad interacting macromolecular networks that occur within cells, i.e. from a Molecular Systems Biology perspective. The availability of sufficient NMR experiment time on the most advanced spectrometers is an absolute prerequisite for state-of-the-art Structural Biology.

In view of the on-going and planned structural genomics / structural proteomics (SG/SP) projects within Europe, the EC found it timely to assess present and future requirements for NMR RIs for SG/SP as well as for Structural Biology *from a users' perspective*. This assessment was carried out in the framework of a Specific Support Action funded by the EC, named the Forum for European Structural Proteomics (FESP, www.ec-fesp.org), established at the beginning of 2006. Its mission is to evaluate requirements for large-scale infrastructures for SG/SP in Europe, including synchrotrons and facilities for NMR spectroscopy, electron microscopy (EM), protein production and bioinformatics. The outcome of the activities of FESP is expected to aid the EC in formulating policies concerning large infrastructures and SG/SP in general.

One major activity of FESP has been the assessment of the existing European bio-NMR RIs, with respect to the requirements of SG/SP projects and of individual SB laboratories, as well as considering future demands. To aid this assessment, a questionnaire was sent to users of bio-NMR RIs at both the European and national levels, as well as to scientists heading NMR research groups throughout Europe, both in academia and in the pharmaceutical and biotech industries.

The study was designed to address the following questions:

- What NMR instrumentation is presently available at European bio-NMR Research Infrastructures, or will become available in the near future?
- What is the current demand for NMR time from SG/SP projects and SB groups, and what are the projected requirements, i.e. is the availability of NMR currently a bottleneck, or is such a bottleneck anticipated?
- Which types of NMR spectrometers and experiments do users require from the bio-NMR RIs and what type of support do they expect to receive?
- What developments do the users foresee in the near future, and what will be their requirements for dealing with such developments?

The findings of the surveys are summarized in this report (Figures 1-16 and Appendices 1-2).

A group of NMR scientists assisted both in the compilation of this report and in the formulation of the conclusions and recommendations to the European Commission. The members of this group were Ad Bax, NIH, USA; Ivano Bertini, CERM, University of Florence, Italy; Rolf Boelens, Bijvoet Centrum, Utrecht University, Netherlands; Iain Campbell, Oxford University, UK; Timothy A. Cross, National High Magnetic Field Laboratory, Tallahassee, USA; Alex Diyky, Norwegian University of Science and

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We would like to thank the members of this group for their advice and constructive criticism. The findings and conclusions of this report, including any errors, remain, however, the full responsibility of FESP.

December 2007

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Introduction

Nuclear Magnetic Resonance spectroscopy (NMR) is the only atomic resolution technique to structurally and dynamically characterize biomolecules in solution. NMR instrumentation, technology and methodology have progressed dramatically over the past two decades and, since the first NMR solution structure was determined in 1985 by Kurt Wüthrich and coworkers, a total of 7000 solution-state NMR structures have been deposited in the RCSB Protein Data Bank (PDB, http://www.pdb.org/) to date (December 2007). Currently, 15% of the structures deposited annually are NMR structures.

The increase in the number of solved solution NMR structures is the result of significant technological advances, which include increased magnetic field strength (the first NMR protein structure was solved with data acquired using a 500 MHz spectrometer, while 900 MHz spectrometers are now widely available), cryogenically cooled probeheads (which can significantly increase sensitivity three-fold and beyond), methodological advances (as examples, the development of 3D and 4D NMR to reduce overlap, use of ^{13}C, ^{15}N and ^{2}H isotope labeling and 3D triple resonance experiments that allow straightforward spectral assignment, and relaxation-optimized sequences like TROSY that now enable NMR investigations of large biomolecules complexes). In addition, improvements in protein expression techniques now enable researchers to routinely produce large amounts of isotope-labeled protein samples for NMR analyses.

The past decade has also seen general advancements in high-throughput protein production techniques, large-scale crystallization trials and automated data collection routines, heralded primarily by Structural Genomics (SG) programs originally established in the late 1990's in the USA and somewhat later in Japan and Europe. These SG initiatives have greatly accelerated the pace of individual protein structure determination processes, and are at the moment responsible for a large portion of the *new or unique* deposited protein structures. At the same time, the number of SB laboratories has increased significantly, and the diffusion of medium- and high-throughput (HTP) techniques from SG consortia into locally applicable laboratory routines has further aided the structure determination process even for single investigator-driven research projects. The recognition of the importance of three-dimensional structure information for a deeper understanding of biological processes, in combination with the development of biological NMR applied to both structural and functional studies as a mainstream molecular biology research tool has led to structural studies being pursued by biologists whose primary expertise does not include NMR spectroscopy. All of these developments have led to an increase in demand for access to bio-NMR RIs.

These trends and the general interests of the structural and molecular biology communities are reflected in the responses given to the survey conducted by FESP in 2006/2007 within the European bio-NMR community. A questionnaire was sent to about 500 users of European and national NMR facilities, and to other NMR spectroscopists, thus covering a large part of the user pool. In total, 160 scientists (32%) returned the questionnaires. Their answers illustrate the broad range of biological questions that are being addressed by users of the bio-NMR RIs. While their primary application is obviously structure determination, they also include the study of protein-protein interactions, protein-RNA/DNA interactions, and protein-small molecule interactions, and in the screening of the latter.

Access to bio-NMR Research Infrastructures does not presently represent a rate-limiting step in the structural and functional characterization of biomolecules in solution. On the contrary, and not surprisingly, protein production and sample optimization still represent the major bottlenecks in biomolecule characterization (35% and 55% of the respondents found these two steps, respectively, to be limiting factors in their research). This finding is understandable, since NMR data collection, as well as the quality of the data collected, strongly rely on obtaining a well-behaved, isotopically labeled sample of a finite concentration, features required for obtaining high-quality NMR spectra.

Funding is another anticipated bottleneck that is "frequently" encountered by 45% of the respondents. With regard to access availability, about 35% of the researchers found that they were "never" limited in

their access to bio-NMR RIs and "never" limited by the process of structure determination and refinement. Although the procedures for NMR structure determination are less automated and standardized than for crystallography, they no longer appear to be a rate-limiting step.

The Impact of NMR on Structural Genomics / Structural Proteomics

NMR spectroscopy has been and continues to be extensively used by a number of SG projects for structure determination. It has the unique advantage of being able to determine the structure in solution; it can therefore be applied to proteins or protein states that are reluctant to crystallize. Furthermore, sample conditions can be varied and adapted to match physiological ones (e.g. pH, temperature, buffer) as closely as possible. The major drawback, however, is the limit in molecular size. Continuous advancements in both hardware and experiments have significantly increased this limitation for routine applications, but high-resolution structures of biomolecules larger than 50kD still remain unobtainable by NMR methods.

Recently, it has been demonstrated that structures of biological macromolecules may be obtained by solid-state magic-angle-spinning NMR. Potentially, this method is useful for determining structures of amorphous or only locally ordered samples, such as amyloid fibrils, proteins attached to natural fibers such as microtubules, the actin cytoskeleton, or membrane protein complexes in a natural membrane environment. Solid-state NMR also has no size limitation. It therefore has the potential to be a method for investigating biological areas that are as of yet underrepresented in the PDB.

NMR structures are generally characterized by a lower apparent resolution with respect to X-ray structures. While this is partially intrinsic to the technique, it also reflects the real situation in solution, where macromolecules continuously sample multiple conformations under physiological conditions. Thus, the apparent lower resolution also reflects existing conformational dynamics, while static crystal structures represent a single state. Therefore, the solution structures and accompanying dynamic information afforded by NMR provide valuable insights into the internal protein mobility and conformational freedom experienced in solution. Both of these properties play key roles in the overall biological function of the characterized proteins.

Sensitive modern NMR spectrometers allow a 2D spectrum of the type of HSQC to be obtained from a 50 micromolar protein sample within 20 minutes, permitting an efficient screen for suitable samples. Crystallization trials and initial diffraction data represent a comparable process, but crystallization screens require hours to months before suitable samples are identified. As one of the fundamental requirements for crystallization is good solution properties, NMR screening can often be used to assess suitable solution conditions for crystallization. NMR can also be successfully used in combination with crystal structures, as NMR structural restraints in solution can be applied to a high-resolution X-ray structure, which can then be optimized to the solution conformation.

An emerging research area of particular interest for NMR spectroscopy, also with regard to structural proteomics initiatives, is the growing number of proteins that do not appear to harbor well-defined structural propensities in their pure states or which contain extensive flexible regions. Bioinformatic analyses have predicted that 7-33% of bacterial proteins contain substantial unfolded sequences; and that 36-63% of eukaryotic proteins contain substantial partially unfolded sequences. A natively unfolded, or intrinsically disordered protein (IDP) state offers significant advantages for recognition by diverse physiological partners and regulatory mechanisms, and indeed they are over-represented in proteins with cellular signaling functions. Moreover, IDPs are implicated in devastating human diseases, including all of the known neurodegenerative amyloid disorders. Fractions of the expressed proteins studied in the framework of an SG/SP program may thus be unfolded, and therefore cannot be expected

to crystallize in the absence of their biological binding partner. This provides a unique opportunity for NMR spectroscopy as simple inspection through NMR spectra can determine if a protein contains extensive unfolded regions and if its interaction with protein partners can induce well-folded functional states in the protein target.

Sophisticated NMR analyses, addressing the local and global dynamic properties of folded and unfolded proteins, can be carried out using relaxation and heteronuclear NOE measurements as well as residual dipolar couplings (RDCs). The intrinsic dynamic properties of biomolecules are important for a wide range of protein functions including catalysis, ligand regulatory interactions, and protein stability. NMR also plays a key role in detecting and characterizing movements of domains relative to one another; such relative domain motions are of special importance when studying multi-domain proteins. Most eukaryotic proteins are comprised of multiple domains often connected by flexible linkers. The relative domain orientations and dynamic changes in their mode of action and biological function play crucial roles in biological processes, such as the regulation of gene expression and signal transduction.

NMR spectroscopy, more than other techniques, is a very powerful tool for investigating protein-protein recognition processes and unraveling complex protein-protein interaction networks, yielding results that are of high impact and of great interest to proteomics, systems biology, and the entire biological community. NMR has a unique potential for detecting weak and/or transient interactions that often play important roles in the regulation of the molecular function of proteins. NMR can also have an impact on other “omics” fields and indeed it has already shown its impact on metabolomics, i.e. the complete description of the metabolite profile of a given organism. It is therefore foreseen that NMR will be of great use in general “systems biology” applications in the near future.

For all these reasons, NMR fulfills a number of key roles in SG/SP studies, and these are likely to lead to an increased demand for access to NMR RIs.

Current European bio-NMR Research Infrastructures

Appendix 2 provides an overview of the currently active European bio-NMR Research Infrastructures, which provide Transnational Access supported by the EC. Five large RIs, located in France, Germany, Italy, the Netherlands and the United Kingdom form a network of distributed RIs. The wide variety of instrumentation available at each of these RIs combines to make an impressive ensemble of high-tech, high-field, and high-sensitivity instrumentation available, with several pieces of equipment providing unique research opportunities. The broadest range of magnetic fields is available at the CERM Magnetic Resonance Center of the University of Florence, where measurements can be made from 0.1 to 900 MHz. The Frankfurt NMR Research Infrastructure features the highest magnetic field presently available (950 MHz). Most of the spectrometers available at the five bio-NMR RIs are equipped with cryogenic probes, cryogenically cooled probeheads. These RIs are at the forefront of NMR technology, and are involved in hardware developments, which are made available to users as soon as they become operational. RI users can perform essentially all of the types of NMR experiments that they can envisage. Access to solid-state NMR spectrometers is particularly relevant, and it is seeing an increasing demand. Solid-state NMR facilities are available in Frankfurt (5 wide-bore spectrometers 400-850 MHz), Florence (wide bore 700 and 850 MHz instruments), and Lyon.

Overall, high-field NMR measurement time equivalent to full-time use of about 3 spectrometers per year is made available free-of-charge to EU users. Each user can choose the optimally configured spectrometers that are best suited for his needs.

At each Research Infrastructure, dedicated staff members are available to assist users with their projects beginning with the initial submission of their individual research proposals. Applications are then evaluated by an international expert panel and, upon approval, included in the internal schedule of each RI. According to the needs of the users, they will be assisted by the RI staff to set up their NMR experiments and to analyze their NMR data. Research Infrastructures can provide access in a variety

of different ways, in accordance with the individual requirements of the users. Users can stay at the RI throughout the entire measurement period, or for an extended period so as to gain experience on data handling and analysis. However, if requested, the user may send a sample in, with RI staff taking care of the measurements, or users may come in only for the initial stages of measurement set-up, after which the RI staff will collect data. Finally, users may come in at a later stage for support in analyzing the results and structure calculation, and to discuss and finalize the results of a project. The staff of each RI handles the administrative procedures relating to the arrangement of accommodations and travel expenses, which are covered by the RIs through the EC Access grant.

A large share of users (over 40%) had access to one of the EC funded Research Infrastructures. Additionally, 40% of the users access national infrastructures, while another 25% access regional infrastructures. This total is higher than 100% as the respondents had the opportunity to mark more than one answer (Fig. 7).

When asked if current bio-NMR access is sufficient for their needs, 58% of the users responded that it was (Fig. 5) and 26% anticipated that it would remain so during the next three years (Fig. 9). However, over two-thirds of the respondents (69%) anticipated an increase in their demand for NMR instrument time over the next three years (Fig. 9). Thus, while the majority of the users found that the present availability of access at bio-NMR RIs meets their current needs, they foresaw an increase in their need for access in the near future. Indeed, a significant number of applications for access are expected to arrive as a result of the widening of NMR applications in Structural Proteomics and in Molecular Systems Biology.

Developments are in progress to reduce the acquisition time for a complete set of NMR data that are required for spectral assignment and NMR data analysis. This may contribute to partially balance the expected increase in access applications.

The requests by users for the development of experiments for more challenging systems and for a wider range of applications, such as lower concentration samples, larger molecular weight systems, and protein-protein and protein-DNA/RNA interactions (Fig. 16), clearly explains the scientific reasons for the high demand for an increase in access to bio-NMR Research Infrastructures. Furthermore, the users require instrumentation that is at the forefront of the available technology as well as specific developments promoted and performed by the bio-NMR RIs.

The general conclusion is that FESP expects that the needs for NMR access time will increase. FESP supports all actions to maintain and support the bio-NMR Research Infrastructures at the forefront of technological and methodological developments and to encourage them to function as active promoters of these advancements.

NMR Instrumentation

One of the drawbacks of NMR spectroscopy is the intrinsic low sensitivity of the technique, as a result of the small population difference between two nuclear spin levels. Low sensitivity is the price that must be paid in order to access such detailed and sophisticated information at atomic level resolution. Consequently, NMR has developed through a constant effort to increase the overall sensitivity of the method. In terms of hardware, this improvement can be achieved through an increase in the magnetic field strength, contributing to an increase in the nuclear energy level difference, which in turn is strongly related to the intensity of the signal. Recently, advancements in NMR hardware technology have produced a reduction in thermal noise associated with different parts of the spectrometer, particularly of the NMR spectrometer probe, which has resulted in another leap in sensitivity. These advancements can be estimated to produce a 4-fold increase in sensitivity, which translates into a 16-fold decrease in the time required for a typical NMR data acquisition procedure.

When respondents were asked which magnetic field they used in the last three years, approximately 38% answered that medium-field instruments (500-600 MHz) were employed, whereas 36% answered that high-field magnets (700-900 MHz) were used. About 17% required access to an instrument with a cryogenically cooled probe in order to study biomolecules at low concentrations, i.e. in the submillimolar range, while 5% requested a solid-state spectrometer.

Access to solid-state NMR spectrometers is particularly relevant, with user requests increasing, as seen as a result of the open question at the end of the questionnaire (What type of experiments would you like to do at bio-NMR infrastructures that cannot be done at present?). Additionally, the request to perform experiments at high field magnets equipped with cryogenically cooled probes is increasing. This points to the fact that the quest for higher sensitivity is still very relevant.

The increase in instrument sensitivity has also opened the way to the study of proteins characterized by an intrinsically low solubility, roughly moving the lower limit from the millimolar to the micromolar range; this has contributed to the dramatic reduction in the experimental time necessary to study systems in the millimolar range. It is now a generally accepted practice to use ^{15}N and ^{13}C isotopic labeling and ^{1}H detection. Another advantage of the great increase in instrumental sensitivity, in conjunction with the use of isotopic labeling, is that not only protons, but also less sensitive nuclei, such as ^{13}C, can be directly detected. This opens up a vast range of additional experiments that provide new observables for systems at the limits of the technology based on ^{1}H detection. Until recently, NMR probes were built with optimized ^{1}H sensitivity, at the expense of ^{13}C sensitivity. Today, and thanks to continuous interactions with European instrument manufacturers, probes with increased sensitivity for ^{13}C have also become available. Further increases in magnetic field strengths together with improvements in probe technologies and other hardware advancements are anticipated in the near future, which will permit studies on even less sensitive nuclei.

Advancements in Methodology

Several new ideas, partially triggered by technological advancements both in sample preparation strategies and instrumentation, have recently had a large impact on NMR and structural biology.

Stable isotope labeling

Many problems inherent to NMR can be solved by the appropriate choices of isotopic labeling scheme, as witnessed in the past by the success of ^{15}N, ^{13}C isotopic enrichment procedures. The latter has now become a routinely accepted and relatively inexpensive technique. Some innovative and ingenious isotopic labeling schemes have recently been proposed and are being consolidated within the community (^{2}H labeling, selective ^{1}H reprotonation of specific groups, such as methyl groups or aromatic residues, ^{13}C “hopping” for solid-state applications, and isotopic enrichment of selected types of amino acids to reduce spectral overlap). It is likely that efforts in this area, in strict synergy with NMR methods development, will further widen the application range of NMR to both proteins and RNA molecules of increasing molecular weight and complexity. Segmental labeling schemes will become essential to increase the size of RNAs to be studied beyond 100 kDa.

Increasing the speed of data acquisition

So-called FAST methods, which reduce the time interval between the acquisitions of two consecutive NMR scans, have recently been proposed for improving sensitivity for a given experimental NMR time. These new approaches contribute to a faster acquisition of NMR experiments that in turn means more pulse cycles, and thus a higher signal-noise ratio in the same amount of time. These experiments already find application in the structure determination process of small-medium size molecules and tools are being developed to make them even more easily accessible and generally applicable. Multiple

detectors are being developed to obtain either an improvement in the signal-to-noise ratio of an experiment by summing/averaging their outputs or to detect different frequencies in the same experiment. Single scan experiments have been proposed that will permit collection of 2D spectra in a single scan, employing gradients. This approach will provide unique insights into fast kinetic processes, thus contributing to the characterization of functional processes.

Molecular size

In order to combat fast transverse relaxation, which becomes a limiting factor in the study of large macromolecules, transverse relaxation optimized spectroscopy (TROSY) methods have been developed for the detection of amide or aromatic proteins, methyl groups, and methylene protons. In the context of new methods for large proteins, ^{13}C direct detection NMR experiments have also been proposed, especially for systems where extensive deuteration is necessary to reduce the large ^{1}H dipolar concentrations to relaxation, resulting in a protein sample devoid of aliphatic ^{1}H. These experiments will definitely contribute to an increase in the size of proteins that can be tackled using NMR. Similar considerations are also true for RNA and DNA molecules.

Automation

Efforts among the NMR community are being directed to establish a general platform that will, in the near future, automate the process of structure determination, from sample preparation through the deposition of NMR structures in the PDB. Such a platform will rely on the development and coordination of a series of steps, ranging from automated peak picking routines to sequence-specific resonance assignment strategies, automatic identification of structural constraints, and structure calculation- and validation procedures.

These automatic procedures will become increasingly reliable as new experiments are developed to access redundant information, such as reduced dimensionality and projection reconstruction methods. This aspect is also synergistic with the FAST methods, which will reduce the amount of time needed to acquire a certain amount of information and thus allow implementation of a larger number of experiments in the same amount of time. Moreover, the automation process will benefit from the incorporation of novel ideas appearing in the literature, which can ultimately be combined to yield generally accepted protocols.

Exploitation of paramagnetic effects

Effects on NMR results deriving from the presence of a paramagnetic metal ion in a protein can provide valuable additional information. In particular, information may be provided on long-range interactions and on the relative orientation of residues in a protein frame or domains. Indeed, beyond taking advantage of the paramagnetic metal ions naturally present in metalloproteins, it is now becoming widely accepted to either substitute diamagnetic metal ions with paramagnetic ones, or to incorporate paramagnetic tags, through protein engineering or chemical modification. As already mentioned, these paramagnetic effects complement existing NMR parameters with long-range information, particularly important for studying protein-protein interactions. The development of standardized methods for sample preparation and new NMR experiments will contribute to making this approach more widely used.

Residual dipolar couplings

Residual dipolar couplings (RDCs), which can be measured by inducing a partial alignment of proteins in solution either with high magnetic fields or with dilute external media in solution, can provide a wealth

of important long range structural and dynamic information. Several endeavors are still in progress to assess the methods and to differentiate between structural and dynamic information content. However there is no doubt about the potential of RDC data to monitor intermolecular interactions and to provide information about the relative orientation of two interacting molecules or of protein domains. Being intrinsically complementary to other structural parameters such as NOEs, RDCs will also contribute to the quality of the deposited NMR structures and to structure validation.

Solid-state methods

Solid-state NMR spectroscopy has recently blossomed with respect to applications to structural biology and to proteins in particular. There are two technologies that have demonstrated great potential; the use of magic angle sample spinning to obtain isotropic spectra and the use of aligned samples for structural protein and membrane protein characterizations. Development of higher fields, special labeling approaches, and faster magic-angle spinning devices now permit acquisition of NMR experiments based on ^{13}C or even ^{1}H direct-detection with high sensitivity and relatively high resolution. These experiments are applicable and actually at their best in, though not limited to, systems with short range order, such as three-dimensional microcrystals, 2D-crystals, and filamentous preparations, filling a gap between X-ray crystallography and solution NMR. In large systems, it can provide complementary information to that which can be obtained in solution and by X-ray crystallography. This area is in rapid development, and the number of structures solved by solid-state NMR is increasing, showing the potentialities of this methodology for the structure determination of insoluble proteins such as membrane proteins or disease-related aggregates – great advances are expected in the near future. Ten protein structures have been characterized by aligned sample solid-state NMR methods and the recent advances made in sample preparation and methodology for the characterization of membrane proteins and transmembrane domains suggest that many structures will be forthcoming.

Multidisciplinary approaches

Strategies are being developed that integrate NMR structural data with those originating from other, complementary techniques in solution, such as small angle X-ray and/or neutron scattering experiments. The potential of integration is also very high with single particle Electron Microscopy and FRET techniques. In such a way a broader range of systems can be structurally characterized in solution, breaking down the molecular size limit and the presence of internal dynamics and/or domain reorientation. NMR structural analysis can therefore also be performed on high molecular weight systems and on multi-domain proteins and complexes, thus opening up new routes for the study of larger complexes in solution.

Research Areas

The current research areas in biomolecular NMR on which major interest is focused are the structure determination of proteins, including protein dynamics and folding, the study of protein-protein complexes, and protein-ligand interactions. Taken together, these areas cover over 65% of likely user interests. All of these areas would profit from key developments such as increased sensitivity, reduction of sample volume, and automation. Further areas of interest for users are DNA/RNA structures and interaction with proteins (4% and 10%, respectively) and target-ligand screening (8%). Solid-state NMR is among the “other areas” (7%), but will certainly become a major player in the future application of NMR to biomolecular systems.

Structure determination of proteins and protein complexes

The contribution of NMR to Structural Biology is presently limited by both sensitivity and spectral resolution. Indeed, improved sensitivity and resolution have always been the two main challenges in biomolecular NMR. A limiting factor is magnetic field strength, B_0. Commercial instrumentation suitable for high-resolution NMR studies currently features magnetic fields of up to 22.3 T (corresponding to 950 MHz proton Larmor frequency). As the signal-to-noise (S/N) ratio increases proportionally to $B_0^{7/4}$ and because the signal-averaging time decreases with increasing the applied magnetic field, the availability of higher-field high-resolution NMR spectrometers is expected to have a dramatic impact on the efficiency of the technique, concurrently broadening the application envelope of NMR analysis. Efforts are being made to produce stable magnetic fields of 23.5 T, i.e. 1 GHz instruments, primarily based on the technology used to produce 22.3 T spectrometers. Attempts are also being made to develop systems utilizing an entirely new technology that involve novel superconductive materials, which would allow the production of 1.2-1.3 GHz spectrometers. Support of these activities on a European level is highly desirable in order to speed up the necessary technological developments.

Until now, the largest protein whose backbone structure has been resolved by NMR is malate synthase G from *Escherichia coli* (723 amino acids, with a molecular weight of approximately 82 kDa). However, currently used routine methods are limited to smaller systems, setting the generally accepted upper threshold for NMR to a molecular weight of 30-35 kDa, equivalent to 250-300 amino acids. Higher dimensionality experiments (4D or higher) are being developed, in conjunction with computational tools for their automated analysis, to expand the potential to larger proteins. The application of these methods would significantly benefit from high magnetic fields, particularly due to improved resolution, which is directly proportional to B_0, with the effect being multiplied by the dimensionality of the NMR spectrum. Improved resolution would be beneficial even when reduced dimensionality or projection reconstruction methods are adopted, due to decreased signal overlap, resulting in enhanced performance of the analysis algorithms.

Conventional high-resolution structure determination by NMR involved a macromolecular sample at millimolar concentrations, in a volume of approximately 500 µl. These numbers can be reduced to 0.2 mM and 200 µl using special tubes and cryogenically cooled probes. It should be kept in mind that a protein concentration of 0.2 mM is close to that of some important proteins in their cellular (or extracellular) environment. If substantial further increases in sensitivity could be achieved, many less abundant biological molecules, with cellular concentrations in the 10-100 µM range, could be studied at their physiological concentration. Labeling schemes that would allow detection of such proteins even when other biomolecules are present are being optimized. This would also have advantages for investigating their interactions with their biological partners. Although significant increases in sensitivity have been obtained in recent years thanks to the increase in magnetic fields, improvements in electronics, and in cryogenically cooled probe technology, further advances are still required for further increases in sensitivity.

Microprobe technology has also been progressing, and sample volumes of 10-20 µl can already be used without producing losses in molar sensitivity. In fact, the use of micro-coils with optimized geometry, with their inherent increased filling factor, can produce a gain in sensitivity relative to conventional probes. Reducing the sample volume is essential in all cases where the total amount of sample is limited (e.g. natural products that are difficult to isolate in large quantities or in the case of costly samples). In addition, small active volumes can be extremely advantageous in flow technology, useful for drug screening. However, the revolution here would be the ability to study small amounts of intact cell cultures.

Reducing volume while maintaining the standard cylindrical sample geometry may also bring advantages to high-resolution studies, for instance in terms of magnetic field homogeneity and better radiofrequency irradiation of the sample.

Overall, many of the possible developments in this area are expected to come from improved and more efficient data acquisition procedures, especially in multidimensional experiments. Proton-detected NMR spectroscopy is limited by the relaxation properties caused either by the size of the biomolecules or by paramagnetic centers encountered in the important class of metalloproteins. Heteronuclear detection may represent a breakthrough that will allow researchers to study larger protein complexes.

Protein dynamics and folding

Protein dynamics is essential for the molecular functions of biomolecules but also plays an important role for protein interactions in regulatory networks. Various tools have been developed in recent years and continue to be developed, including relaxation dispersion experiments and approaches to derive domain dynamics from RDCs.

The study of reversible dynamic processes such as protein folding requires the possibility of changing sample conditions in a relatively short period of time. Indeed, the use of NMR to study a large variety of physical/chemical/biological phenomena requires such a capability. Research into the molecular changes occurring in a reversible process can provide valuable insights into the mechanisms of specific recognition and interaction processes, and into the driving forces for various reactions, including protein folding. Thus, developments in ancillary equipment and automation that will permit rapid changes in experimental conditions are desirable.

Furthermore, studies of diffusion and transport processes are expected to provide answers to specific and important questions. Increased sensitivity of biomolecular NMR measures, accompanied by a concomitant decrease in sample volume, would allow the use of higher gradient strengths (making use of shielded gradients) than is currently possible.

DNA/RNA structure and protein/nucleic acids interactions

It is increasingly recognized that RNA plays an essential part in cellular processes including transcriptional control, protein synthesis, and RNA splicing; in addition, many non-coding RNAs have been shown to be involved in brain development, cell fate (micro-RNAs) and epigenetics. NMR spectroscopy allows the structure and dynamic characterization of RNAs and their complexes when crystallization fails. In particular, RNA conformation switching between multiple functional states is a key aspect of the regulatory mechanism in prokaryotes and eukaryotes.

Screening of small molecule libraries

Small molecule interaction screening has become an increasingly important area in biomolecular NMR. NMR has long been used to detect the binding of small molecules to bio-molecular targets, but high-throughput NMR techniques for studying molecular interactions in solution have only recently begun to emerge. A variety of well-known NMR techniques have been used for screening, including nuclear Overhauser effect, chemical shift perturbation, diffusion, relaxation and saturation transfer, which taken together provide an extremely powerful set of tools for studying the interactions of small molecules with proteins. However, screening of small molecule libraries, in order to be broadly useful, requires further increases in throughput. There is therefore a need for the development of automated, high-speed sample changers. At the same time, the continuous increase in magnetic field strengths poses technical challenges to the construction of such automated devices.

Solid-state NMR

In recent years, solid-state NMR spectroscopy for structural studies of macromolecules such as membrane proteins has rapidly advanced and continues to do so. Indeed, developments in

instrumentation (high fields, improved probe technology, fast Magic Angle Spinning (MAS), low electric field NMR probes that minimize sample heating, experimental approaches (new NMR experiments to facilitate sequence-specific assignment and collection of structural restraints), data analysis (structural information from chemical shifts, for example), isotopic labeling techniques (various schemes to suppress one-bond C-C interactions, ^{2}H isotopic enrichment, ^{1}H isotopic dilution, etc.) now permit the acquisition of high-resolution, high-sensitivity solid-state NMR experiments. For samples of moderate signal complexity in terms of the expected number of resonances, these new procedures actually compete with the analogous solution NMR experiments. As a result of these developments, several 3D structures determined using MAS solid-state NMR have already appeared, the first in 2002. In principle, solid-state NMR is not limited by molecular size, one of the limiting factors in solution NMR structural studies, and can thus offer valuable information about large biological macromolecules, such as membrane proteins which are inherently difficult to crystallize or large molecular aggregates, especially those involving cytoskeletal proteins. For membrane proteins, whose structures are often highly dynamic, have multiple conformations and are sometimes structurally heterogeneous, solid-state NMR has great potential. Recent advances in aligned sample preparations using mechanical alignment on glass slides and magnetic alignment of bicelles has suggested that a wide range of membrane proteins, including GPCRs, can be characterized by solid-state NMR. Additionally, paramagnetic effects in the solid-state constitute a very promising way of providing additional information in solid-state NMR.

In-cell NMR

The recent development of high-resolution NMR methods for the observation of proteins inside live cells represents a new area of biomolecular NMR spectroscopy. In-cell NMR spectroscopy enables structural and functional investigations of biological macromolecules in intact cells and thereby allows for non-invasive, non-disruptive *in situ* studies of complex cellular processes. Unparalleled by any other biophysical technique, in-cell NMR spectroscopy can be employed to decipher diverse structural protein conformations inside live cells, or to determine varying functional *in vivo* propensities of proteins at different cellular conditions.

These types of analyses are particularly important for the advancement of our understanding of human neurological disorders, such as amyloid diseases for example, as well as to further our knowledge about the functional *in vivo* consequences of post-translational protein modifications.

Trends and Future Developments

NMR is the only technique that allows us to study the structural and dynamical properties of macromolecules and their interactions, often weak and transient, at the atomic level in solution. A price paid for the wealth of information is the intrinsically low sensitivity of the technique that limits the size of the systems that can be studied. Fortunately, the impressive technological improvements achieved in recent years (in particular high B_0 and cryogenic probe technology) have contributed to a dramatic increase in instrumental sensitivity.

For these reasons NMR is positioned to play a central role in the new challenges presented by systems biology. The continuous development of new instrumentation and new methodologies has greatly expanded the range of applications of NMR spectroscopy to molecular systems that, only a decade ago, could not be studied with this experimental technique.

NMR users' survey

In the survey submitted to NMR users, they were asked for their views on desirable future developments at bio-NMR RIs, and asked about the type of experiments they would like to perform at NMR spectrometers that cannot be carried out at present (Figs. 15 and 16). The highest priority was given to

various improvements (biomolecular solid-state NMR, NMR of large systems, $^{13}C/^{15}N$ direct detection, high-field measurements using cryogenic probes, studies on dynamics, biomolecular interaction studies) and their suggestions are summarized below. Many of these suggestions have already been implemented, or are in the process of being implemented, and others are under development. It may be appropriate here to emphasize the importance of EC support for such methodological developments. An example is the BioDNP (Dynamic Nuclear Polarization) project, presently supported through the Infrastructure Division, which aims at enhancing NMR intensity via dynamic nuclear polarization, which is very challenging but could have a dramatic effect on biomolecular NMR applications.

Need for access to bio-NMR Research Infrastructures

Among those who responded to the NMR questionnaire, 70% ranked access to bio-NMR Research Infrastructures as essential for their research, and 18% ranked it as important (Fig. 4). While 64% considered the present access to bio-NMR RIs as sufficient for their present needs (Fig. 5), almost 70% foresee an increased need for such access within the next 3 years (Fig. 9).

These responses represent strong and compelling requests for national and European funding bodies to increase the level of support for access to bio-NMR RIs not only in terms of instrument time, but also with respect to instrumentation features and advanced methodology. The strategic importance of such access is reflected in the share of publications that rely on experiments performed at bio-NMR RIs. For approximately 40% of the respondents, over 70% of their peer-reviewed publications rely on their access to bio-NMR RIs (Fig. 10).

User support

Figures 14 and 16 clearly indicate that user support by bio-NMR RI scientists is a key element in increasing the relevance and impact of research access. 69% of the survey respondents stated that local support by NMR scientists is essential, and ensures efficient use of precious NMR spectrometer time. For an additional 30% of the users it is important. Local support is assessed to have much higher priority than training by specially organized courses, which are nevertheless considered as essential by 37% and important by 56% of respondents. User support often requires a significant commitment from the bio-NMR RI staff, and it is vital that career paths for NMR scientists are allowed to develop in such a way that activities such as user support are given sufficient credit. In addition, it must be ensured that NMR scientists working at the NMR Infrastructures have sufficient time to perform their own research, as the development of novel methods, experiments, and technologies is a key aspect for the quality and impact of the access. Indeed, in this way the users would benefit not only from access to the state-of-the-art instrumentation but also from the most advanced methodologies. There is also a strong demand for training courses devoted to users of bio-NMR Research Infrastructures, which were ranked as essential by 35% and important by 62% of the respondents.

Travel support / Mail service / Remote data collection

Support for travel to the bio-NMR Infrastructures is essential/important for approximately 80% of users. The user survey also shows that remote access with shipment of the sample and data acquisition by the NMR Infrastructure staff is not the highest need, at variance with what is required within the crystallographic community. This can be explained by the fact that NMR experiments are less standardized with respect to synchrotron beam line data acquisition. For NMR data acquisition, a user needs to control the status of the sample and to adapt the settings of the various NMR experiments – all these steps are best optimized through close contact between the user who knows the biological system, even if not necessarily the NMR technique, and the staff scientist at the Research Infrastructure who is an expert with the advanced NMR instrument.

It is now possible to remotely record NMR spectra at the Research Infrastructures by sending in the samples and a list of the requested experiments and their setup. This "mail service" use of infrastructures currently occurs through dedicated, experienced technical staff who are in close (e-mail, messenger) contact with the user. As mentioned and rationalized above, this option is not currently considered as particularly important by the BioNMR user community. Implementation of genuine remote access through the web may become available in the near future but, considering that the NMR equipment is very sophisticated and that each instrument requires special care, measurements will still require close coordination with and surveillance by a member of the RI staff.

Thus, while remote data collection is possible, travel to the Research Infrastructure will remain necessary for the majority of users.

Automation

Automation of the available protocols and programs available for structure determination, starting from acquisition and analysis of NMR experiments, to data processing, to sequence specific assignment, collection of structural constraints and finally structure calculation and validation is one of the most important aspects currently being pursued. The wide variety of approaches available in the literature needs to be integrated in a coordinated platform to allow users to employ them in a user-friendly way.

Equipment and hardware advances

The role of bio-NMR RIs is to provide specific expertise and instrumentation for challenging applications. Therefore, it is important to continue to update and improve the instrumentation and methodologies to maintain the RIs at the "cutting edge". In this respect, from the users' point of view, there is a strong demand for spectrometers with increased sensitivity and the ability to tackle samples of higher complexity and molecular weight. The former aspect, i.e. increased sensitivity, can be addressed, as requested by a number of users, by implementing cryogenic probes in various configurations. For the latter aspect, there is a compelling demand for ultra-high magnetic fields. There is also significant interest and expectation for how the DNP techniques will develop. Finally, there is a significant demand for progress in implementing solid-state NMR, particularly with respect to increasing the speed of sample rotation. Breakthroughs in the near future should therefore be with respect to higher magnetic fields, cryo-technology for probes and detectors, and very high-speed spinning rotors for solid-state NMR.

Methodological advances

The developments expected in the near future focus on several aspects related to methodology and protocols for data analysis and structure determination, and to the logistics of access offered by the existing bio-NMR RIs. As far as methodology is concerned, NMR is a relatively new technique compared to X-ray crystallography, and is thus in a phase where new ideas frequently appear in the literature related to new observables, new procedures, and new methods for improving the tools both for structure determination and for dynamic characterization of biological macromolecules and their interactions. One of the roles of the Research Infrastructure is to latch on to these new ideas and to convert them into easily applicable protocols to make them readily accessible to the scientific community as a whole in a timely fashion.

The types of NMR experiments performed by the users of the bio-NMR Infrastructures in the past 3 years reflect both the types of studies and the systems that they are investigating and they plan to investigate in the next 3 years. The majority of the users exploit bio-NMR RIs for protein structure determination (65%), for characterizing protein-protein interactions (60%) and for studying and screening protein-ligand interactions (approximately 70%). The most requested methodological

developments and advancements are closely linked to these types of investigation. There is a need to reduce the acquisition time not only to allow more efficient use of NMR time but also for studying unstable proteins. For this reason, advances in fast acquisition and reduced dimensionality experiments are sought after. The users also see the need for further developments in protonless NMR, i.e. experiments that are based on ^{13}C detection rather than on ^{1}H detection. Using this approach, systems that experience broad ^{1}H resonances or reduced chemical shift dispersion, as is the case for unfolded proteins, can be characterized, since heteronuclear signals experience much larger chemical shift dispersions and are less affected by phenomena that induce line broadening. There is also a demand for new experimental approaches to improve protocols (FAST, RDCs, paramagnetic tags, improved isotopic labeling strategies, etc.).

Standardization

There are a wide variety of experiments, formats, and protocols in use by the NMR community. This has the positive feature of resulting in novel solutions to problems encountered. On the other hand, this variety of techniques does not help to make the many novel features readily accessible to the non-experienced user of bio-NMR. Therefore, from the user perspective, it is highly desirable to have access to universally accepted protocols and data formats that facilitate the use of sophisticated techniques. This, in turn, would permit challenging biological problems to be addressed by scientists who do not necessarily wish to become experts in the field. Several efforts are indeed focusing on this issue, and remote access to calculation platforms should be available in the near future. A new project funded by the EC in the frame of the electronic infrastructures has recently started (eNMR, project no. 213010). One of the main objectives of the project is to provide an integrated, standardized calculation platform for applications in bio-NMR. The platform will exploit Grid technology, and will become available for the users of the NMR Research Infrastructures. A national on-going initiative toward the standardization of data formats is the CCPN project (*http://www.ccpn.ac.uk/*), which is intended as a standard for data exchange between different programs and databases.

Conclusions and Recommendations

On the basis of this survey and discussions with a number of leading scientists in the field of bio-NMR, FESP recommends the following actions to the European Commission:

Essential role of bio-NMR Research Infrastructures

Bio-NMR Research Infrastructures equipped with the most advanced instrumentation are crucial for the success and competitiveness of European Structural Biology. These infrastructures have been and continue to be used extensively, by structural biologists as well as by biologists in general; these scientists are exploiting the wide-ranging potential of NMR for the characterization of biological molecules and processes. Access to these large-scale facilities has been supported by the European Commission since 1994, and this support has been absolutely vital for the success of European Structural Biology. FESP strongly recommends continued funding of access to bio-NMR facilities as large-scale infrastructures of vital importance for internationally competitive biological research. Funding under the access scheme for large infrastructures should include direct support to users for travel (including sample shipment), and support to the NMR facilities themselves for technical staff and running costs.

Number of bio-NMR Research Infrastructures

There are presently five such bio-NMR Research Infrastructures funded by the EC which provide Transnational Access (Appendix 2). These Research Infrastructures are networking with other smaller, regional facilities in such a way as to address all the needs of users and to optimize access (a wide network with a balanced geographical distribution) and the wide availability of the most powerful cutting-edge instrumentation (at the large RIs). Together, the current bio-NMR RIs are adequate to meet the present needs of the bio-NMR community, both in terms of instrumentation time and the available types of equipment. It is, however, foreseen that the demand will increase dramatically in the near future, as NMR can be applied not only to structural determination but also to a wide range of applications in the characterization of biological macromolecules, such as the characterization of weak, transient interactions between biological macromolecules, screening studies of active pharmaceutical ingredients and of metabolites. The demand for NMR time is also going to increase markedly as the availability of samples for membrane proteins increases, thus increasing the needs of access to solid-state NMR.

From this broad range of applications, the considerable potential of NMR as a key technique for studies within Molecular Systems Biology initiatives is clearly emerging.

Procedures for the generation of automated NMR data acquisition routines should be enforced

High priority has to be given to the development of automation in NMR data acquisition, handling, and analysis. Efforts to allow better data analysis should be particularly encouraged, including automatic resonance assignment. Automation should also be supported by implementation of remote access. This would ultimately reduce the amount of user travel to the NMR Research Infrastructures, but its implementation will require investments in NMR scientists and technical staff for development of automated procedures, for data acquisition and analysis.

NMR method development should be strengthened

Development and implementation of new technologies for macromolecular characterization in solution by NMR are essential for maintaining the competitiveness of European structural biology in both academia and industry. This includes higher magnetic fields, improved sensitivity, further exploitation of cryogenic probe technology, and advanced techniques for very large proteins, fast acquisition, very high-speed magic angle spinning (MAS) for solid-state NMR, and automation for peak picking, and resonance and NOE assignment. These developments should be coordinated and supported at the European level, and FESP strongly recommends that the European Commission funds projects devoted to the achievement of these objectives.

Appendix I – FESP Survey of the BIO-NMR RESEARCH INFRASTRUCTURES USERS IN BIOMOLECULES CHARACTERIZATION

A. Survey group profile

Figure 1: Years as principal investigator and research environment of the 160 survey respondents (percentage of answers).

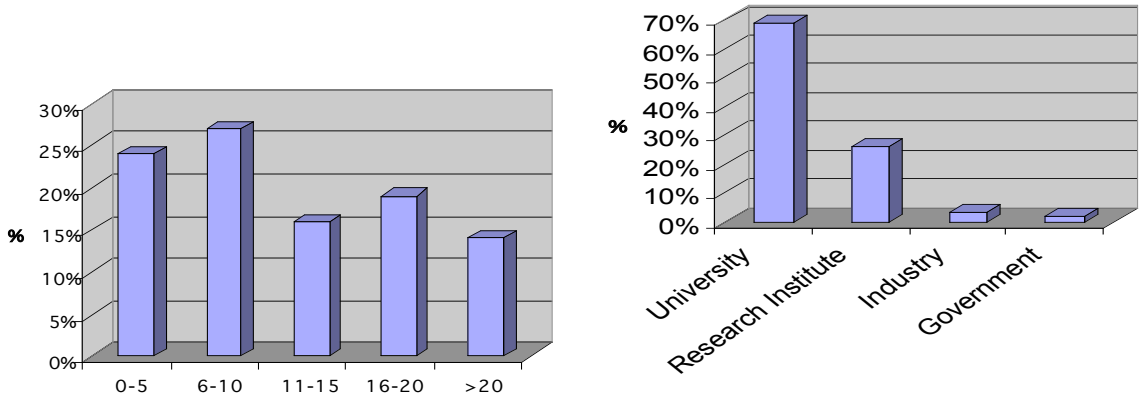


Figure 2: Major sources of research grants (% of answers) of the survey respondents.

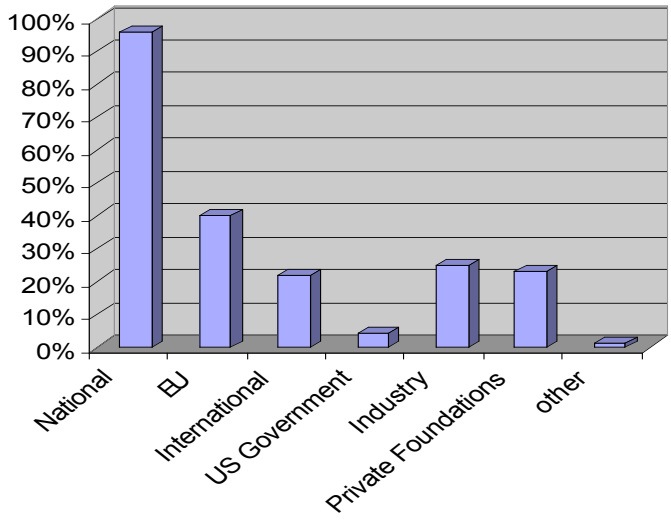
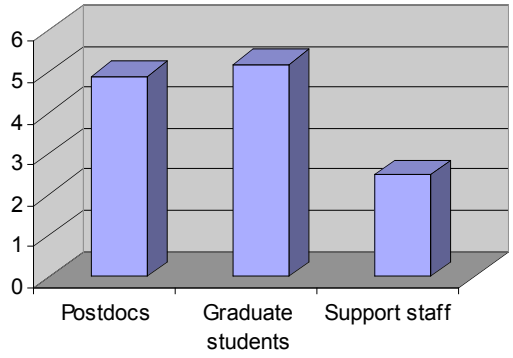


Figure 3: Average number of postdoctoral students, graduate students and support staff per respondent.



B. Questionnaire responses

How important is access to NMR facilities for your research?

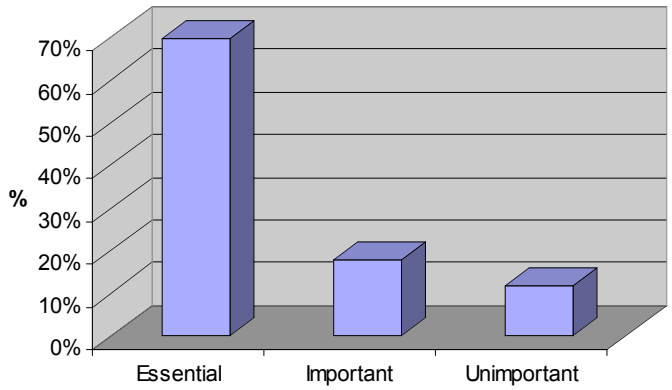


Figure 4

Is present access to NMR instruments sufficient for your needs?

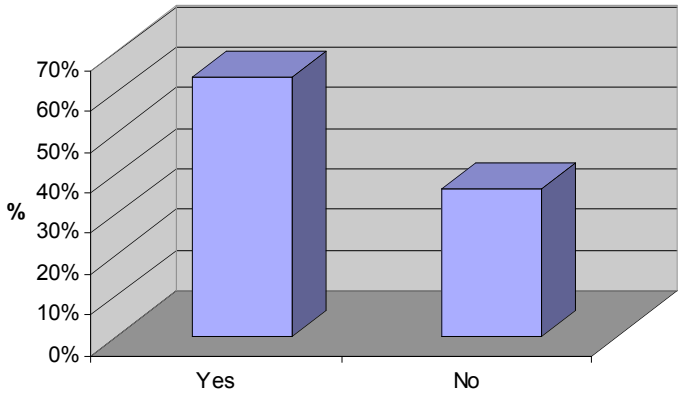


Figure 5

Please estimate how many days of NMR time at a bio-NMR RI your group has used over the past 3 years.

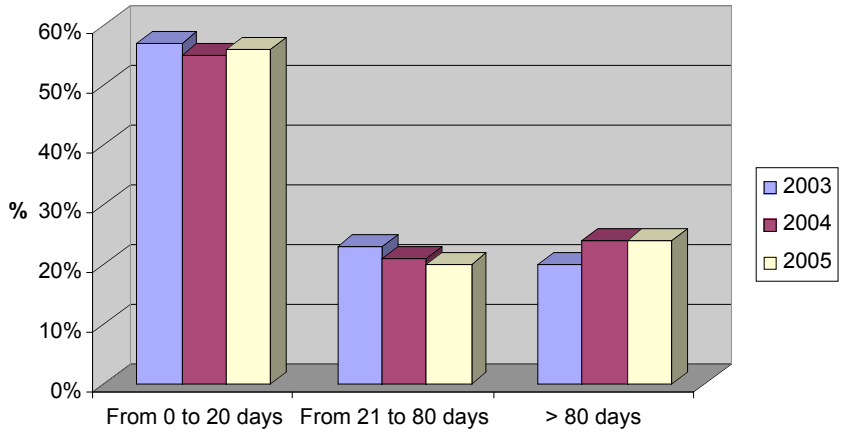


Figure 6

Which type of bio-NMR RI have you been using during the past 3 years?

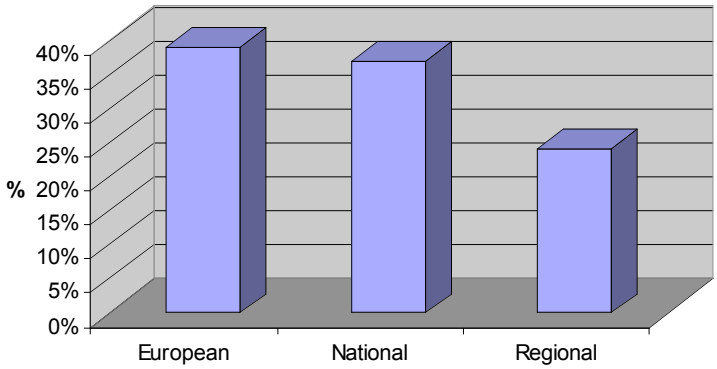


Figure 7

What magnetic fields have you used in your research during the past 3 years?

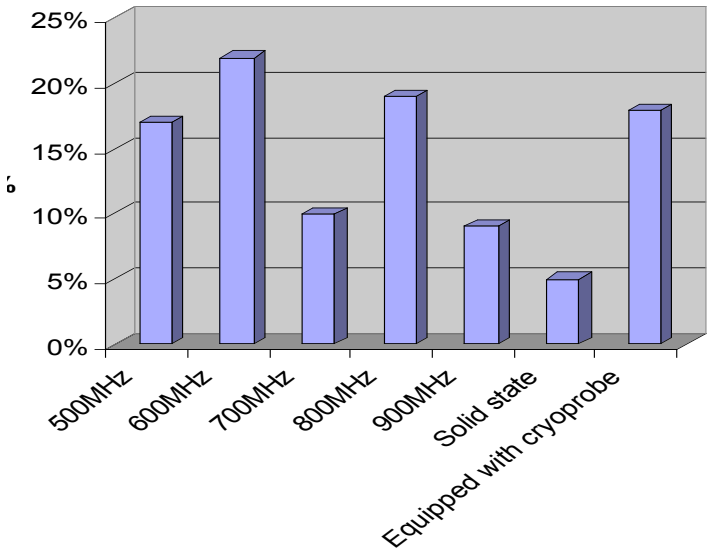


Figure 8

How will your needs for access to bio-NMR RIs change during the next 3 years?

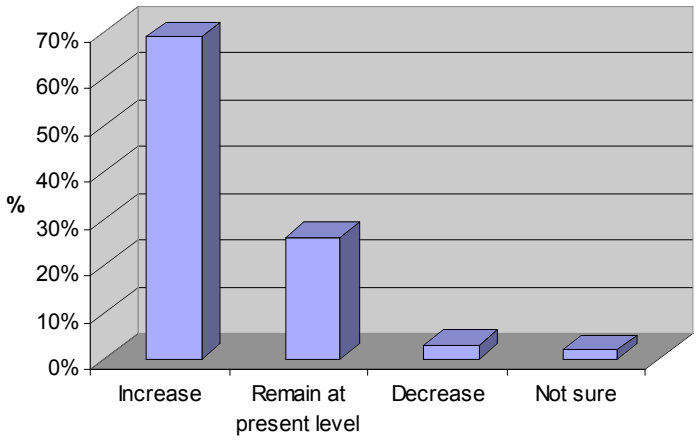


Figure 9

What percentage of your total peer-reviewed publications rely on access to BioNMR RIs?

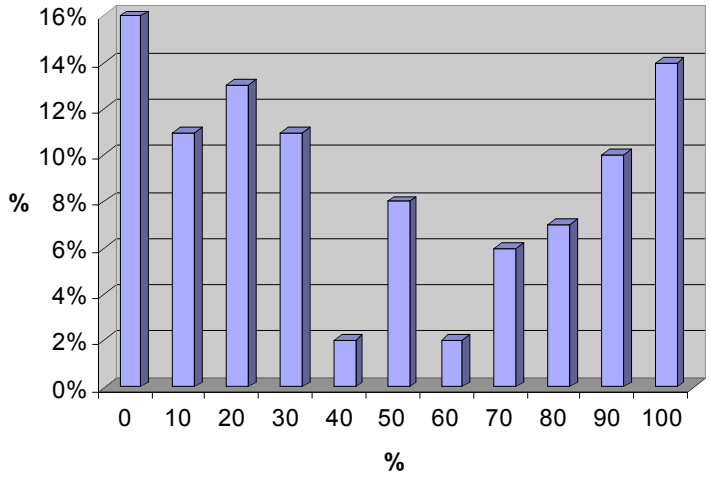


Figure 10

What type of experiments have you been doing on NMR spectrometers during the past 3 years?

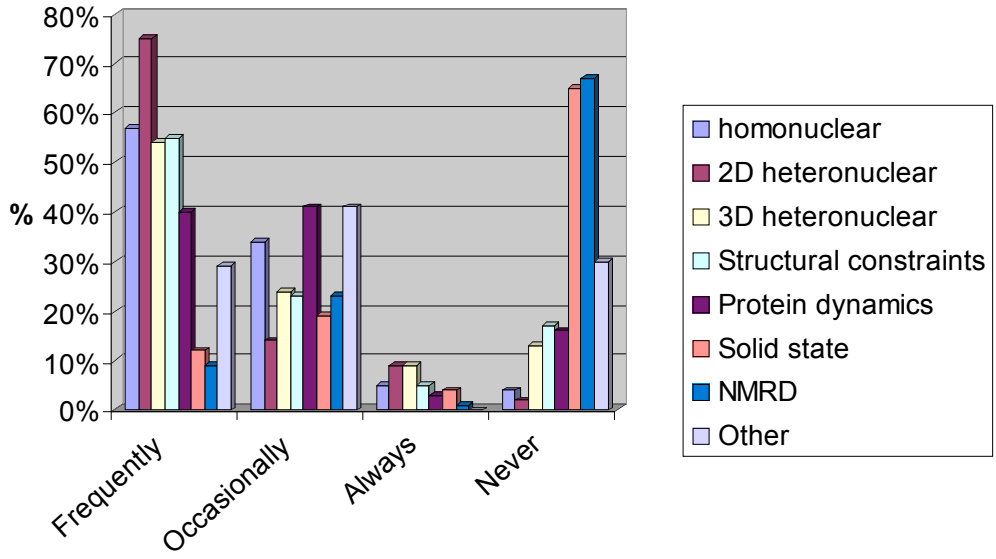


Figure 11

Which of the following will be important uses of bio-NMR RIs for your research during the next 3 years?

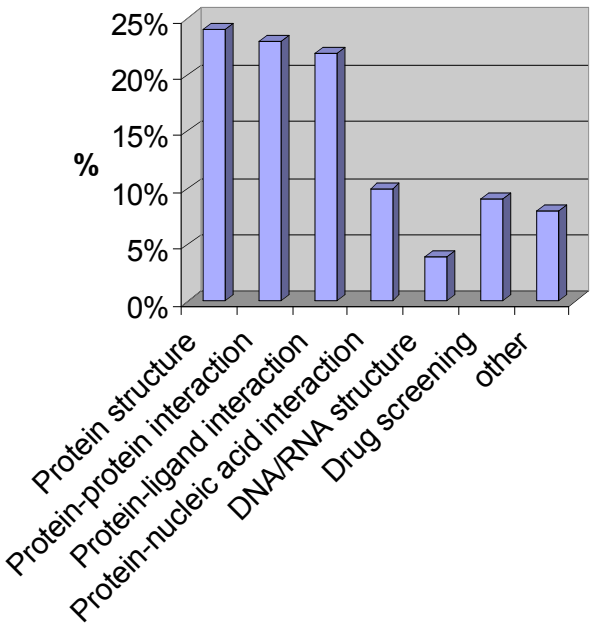


Figure 12

What are the rate-limiting steps in your research at present?

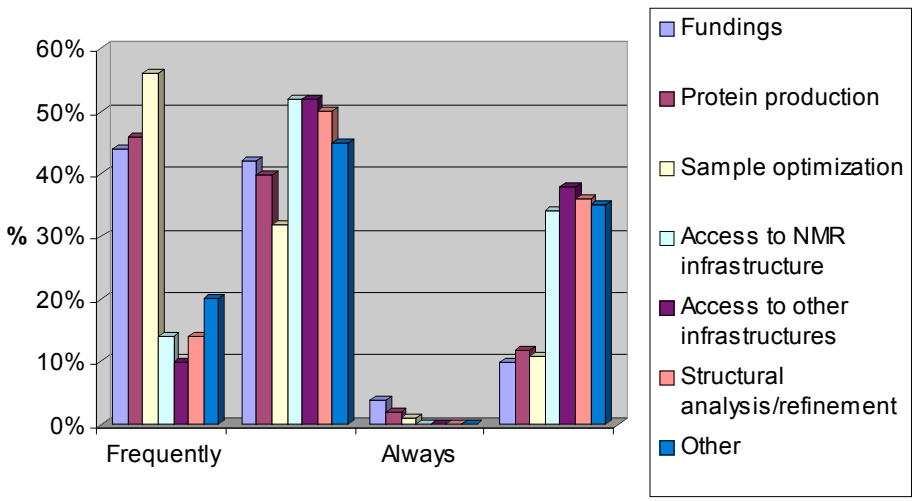


Figure 13

Please indicate the significance given to the training and education of users.

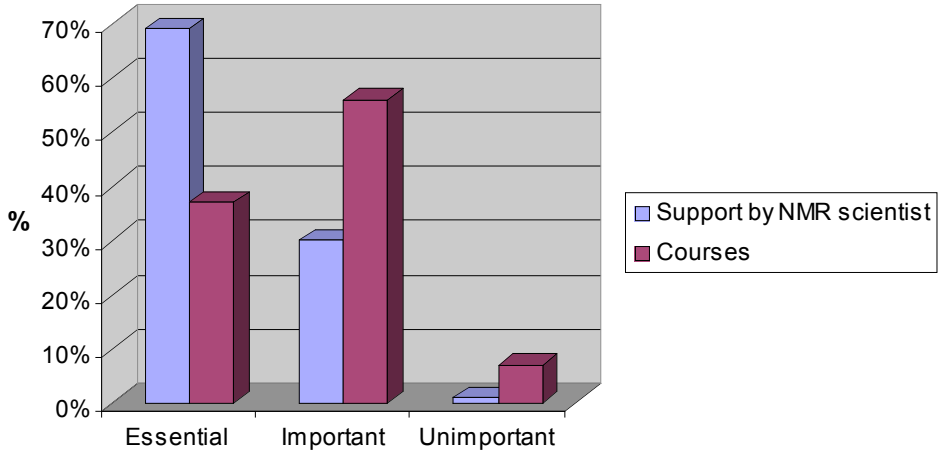


Figure 14

How important for you is travel support for the use of BioNMR RIs?

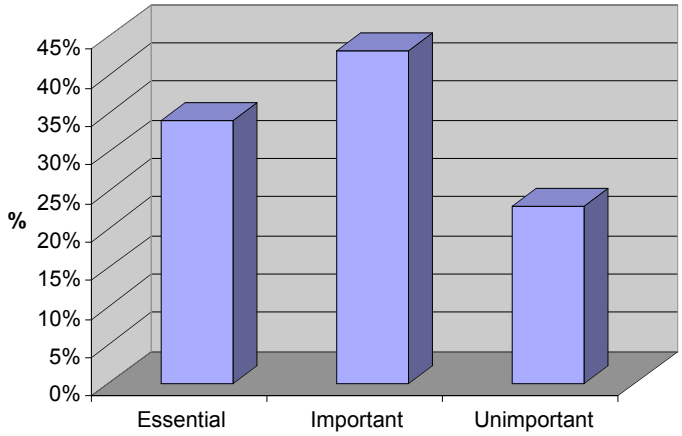


Figure 15

What developments would you like to see at BioNMR infrastructures in the near future?

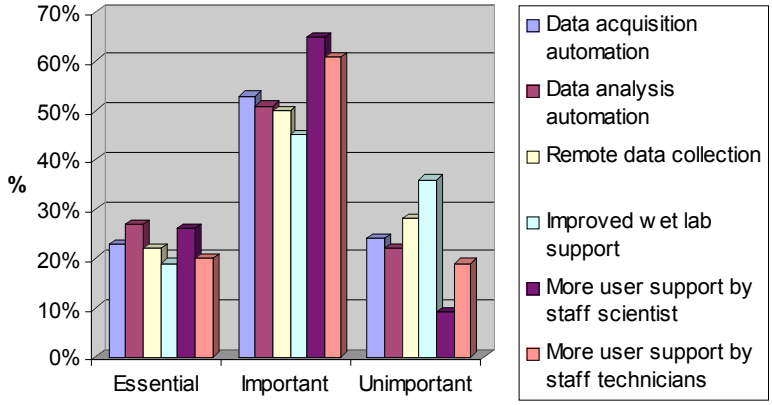


Figure 16

What type of experiments would you like to do at BioNMR Ris that cannot be done at present?

- NMR experiments on large systems (100 KDa at least) (**4**)
- Ligand screening experiments (**1**)
- Development of specific pulse sequences with support staff (**1**)
- Advances in solid-state NMR (**8**)
- Availability of multiple fields at the same time (**2**)
- Advanced techniques for dynamics studies (**4**)
- Developments of $^{13}C/^{15}N$ direct detection (**4**)
- DNP technologies (**3**)
- Heteronuclear 3D experiments (^{13}C, ^{31}P,^{15}N, ^{1}H) for protein/DNA or protein/protein complexes and lipid/protein interactions (**3**)
- High pressure protein NMR (**1**)
- High field NMR (900-800 MHz) experiments with cryoprobes and high sensitivity for low concentration samples (**4**)
- Fast acquisition (**1**)
- High field relaxometer (**1**)
- Protein folding (**1**)
- ^{1}H spinlock experiments at lower field with cryoprobes (**1**)
- RNA pulse sequence optimization (**1**)
- ^{13}C natural abundance experiments (**1**)
- Redfield's NMR sample shuttler (**1**)

APPENDIX 2: EUROPEAN BIO-NMR RESEARCH INFRASTRUCTURES PROVIDING TRANSNATIONAL ACCESS AND AVAILABLE INSTRUMENTATION

CIRMMP (CERM) - Consorzio Interuniversitario Risonanze Magnetiche di Metalloproteine Paramagnetiche & Magnetic Resonance Center Florence, Italy

Spectrometer	Sample state	Cryogenic probes available	Probes available	Channels available
900 MHz (Bruker Avance II)	Solution	5 mm TCI cryo ^{1}H-^{13}C/^{15}N-^{2}H Z-GRD	5 mm TXI ^{1}H-^{13}C/^{15}N-^{2}H Z-GRD	Three + ^{2}H decoupling.
800 MHz (Bruker Avance DRX)	Solution	5 mm TXI cryo ^{1}H-^{13}C/^{15}N-^{2}H Z-GRD	2.5 mm HP (SEL ^{1}H/HP) prototype dedicated, 5 mm TXI ^{1}H-^{13}C/^{15}N-^{2}H Z-GRD, 5 mm TXI ^{1}H-^{13}C/^{15}N-^{2}H XYZ-GRD, 5 mm QXI ^{1}H-^{31}P/^{13}C/^{15}N XYZ-GRD	Four + ^{2}H decoupling
850 MHz (Bruker Avance III)	Solid state (Wide Bore)		3.2 mm MAS DVT ^{15}N/^{13}C/^{1}H	Three
700 MHz (Bruker Avance II)	Solid state (Wide Bore)		3.2 mm MAS, 4 mm MAS	Three
700 MHz (Bruker Avance AV II)	Solution	5 mm TXO cryo for direct ^{13}C detection ^{13}C/^{15}N-^{1}H-^{2}H Z-GRD	TXO probe for direct ^{13}C detection ^{13}C/^{15}N-^{1}H-^{2}H Z-GRD	Four + ^{2}H decoupling
700 MHz MHz (Bruker Avance DRX, with standard BACS120 autosampler)	Solution	5 mm TCI cryo ^{1}H-^{13}C/^{15}N- ^{2}H Z-GRD	5 mm TXI ^{1}H-^{13}C/^{15}N-^{2}H Z-GRD, 5 mm TXI ^{1}H-^{13}C/^{15}N-^{2}H XYZ-GRD	Three + ^{2}H decoupling
600 MHz (Bruker Avance DRX with 40A Grad. Ampl. for diffusion measurements)	Solution		5 mm TXI ^{1}H-^{13}C/^{15}N-^{2}H Z-GRD, 5 mm SEL ^{1}H, 5 mm SEL ^{1}H/HP, 5 mm BBO, 10 mm BBO, 10 mm BB (LowFreq), 5 mm BBI	Three
600 MHz (Bruker Avance II, with standard BACS120 autosampler, Bruker liquid handler system)	Solution	5 mm TCIP cryo ^{1}H-^{31}P/^{13}C-^{2}H Z-GRD with ATMM	5 mm TXI ^{1}H-^{13}C/^{15}N-^{2}H Z-GRD with ATMM	Three + ^{2}H decoupling
500 MHz (Bruker Avance DRX)	Solution	5 mm TCI cryo ^{1}H-^{13}C/^{15}N-^{2}H Z-GRD	5 mm TXI ^{1}H-^{13}C/^{15}N-^{2}H Z-GRD, 5 mm BBI	Three

400 MHz (Bruker Avance DPX)	Solution		5 mm BBO Z-GRD, 5 mm SEL ^{1}H for paramagnetic spectroscopy	Two
Relaxometers	**Field Range**			
FFC Stelar	10 kHz - 40 MHz			
90 MHz Bruker	4 - 90 MHz			

JWGU-BMRZ (BMRZ) - J.-W. Goethe-Universität Center for Biomolecular Magnetic Resonance Frankfurt am Main, Germany

Spectrometer	Sample state	Cryogenic probes available	Probes available	Channels available
950 MHz (Bruker _AvanceIII)	Solution	5 mm TCI cryo $^{1}H,^{15}N,^{13}C$ Z-GRD	5 mm TXI $^{1}H,^{15}N,^{13}C$ XYZ-GRD	Four
900 MHz (Bruker Avance)	Solution	5 mm TXI cryo $^{1}H,^{15}N,^{13}C$ Z-GRD	5 mm TXI $^{1}H,^{15}N,^{13}C$ XYZ-GRD 5 mm TXI $^{1}H,^{13}C,^{31}P$ XYZ-GRD 5 mm TXI $^{1}H,^{15}N,^{13}C$ Z-GRD	Six
800 MHz (Bruker Avance)	Solution	5 mm TXI cryo $^{1}H,^{15}N,^{13}C$ Z-GRD	5 mm TXI $^{1}H,^{15}N,^{13}C$ XYZ-GRD 5 mm QXI $^{1}H,^{13}C,\ ^{15}N,\ ^{31}P$ XYZ-GRD 5 mm TXI $^{1}H,^{15}N,^{13}C$ Z-GRD	Four
800 MHz (Bruker DRX)	Solution	-	5 mm TXI $^{1}H,^{15}N,^{13}C$ Z-GRD	Four
700 MHz (Bruker Avance)	Solution	5 mm TXI cryo $^{1}H,^{15}N,^{13}C$ Z-GRD	5 mm TXI $^{1}H,^{15}N,^{13}C$ Z-GRD	Three
600 MHz (Bruker AvanceIII)	Solution	5 mm TXI cryo $^{1}H,^{15}N,^{13}C$ Z-GRD, 5 mm TCIP cryo $^{1}H,^{31}P,^{13}C$ Z-GRD	-	Four
600 MHz (Bruker AvanceII)	Solution	5 mm TXI cryo $^{1}H,^{15}N,^{13}C$ Z-GRD	5 mm TXI $^{1}H,^{15}N,^{13}C$ XYZ-GRD	Four
600 MHz (Bruker DRX)	Solution+ MAS	-	5 mm TXI $^{1}H,^{15}N,^{13}C$ XYZ-GRD 5 mm TXI $^{1}H,^{13}C,^{31}P$ Z-GRD 5 mm TXI $^{1}H,^{15}N,^{13}C$ Z-GRD MAS- TXI $^{1}H,^{15}N,^{13}C$ Z-GRD	Four
600 MHz (Bruker DRX with standard BACS120 autosampler)	Solution	-	5 mm TXI $^{1}H,^{15}N,^{13}C$ Z-GRD	Four
500 MHz (Bruker Avance II)	Solution	-	5 mm TXI $^{1}H,^{15}N,^{13}C$ XYZ-GRD	Four
500 MHz (Bruker DRX)	Solution	-	5 mm TXI $^{1}H,^{15}N,^{13}C$ XYZ-GRD	Four
400 MHz (Bruker Avance with standard BACS120 autosampler)	Solution	-	5 mm TXI $^{1}H,^{15}N,^{13}C$ Z-GRD 5 mm BBI ^{1}H,BB Z-GRD	Three
WB-850 MHz (Bruker AvanceIII)	Solid state (Wide Bore)		4mm TXI CP MAS $^{1}H\{^{13}C/^{15}N\}$ 3.2mm TXI CP MAS	Three

			$^{1}H\{^{13}C/^{15}N\}$ 3.2mm BBI CP MAS $^{1}H\{BB\}$	
WB-600 MHz (Bruker Avance)	Solid state (Wide Bore)		4mm TXI CP MAS $^{1}H\{^{13}C/^{15}N\}$ 4mm CP MAS $^{1}H\{^{13}C\text{-}^{31}P\}$ static probe flat coil $^{1}H,^{13}C,^{15}N,^{31}P$	Three
WB-400 MHz (Bruker AvanceII)	Solid state (Wide Bore)		4mm TXI CP MAS $^{1}H\{^{13}C/^{15}N\}$ 7mm CP MAS $^{1}H\{^{13}C\text{-}^{31}P\}$ static probe flat coil $^{1}H,^{13}C,^{15}N,^{31}P$ solenoid coil $^{1}H,^{31}P$	Three
WB-400 MHz (Bruker Avance)	Solid state (Wide Bore)		7mm TXI CP MAS $^{1}H\{^{13}C/^{15}N\}$	Three
Bruker Elexsys E580 Spectrometer	EPR		Operating frequency 9-12GHz cw-EPR, pulsed EPR, ENDOR and PELDOR	
EPR S-band homebuilt	EPR		Operating frequency 2.7 and 3.7 GHz, pulsed EPR	
G 180GHz homebuilt	EPR		180 GHz, EPR	
ESP300 X-band (Bruker)	EPR		parallel mode EPR and including ENDOR-type	

RUUTR.BCBR (SONNMR LSF) - Universiteit Utrecht Dept. of Chemistry, Section NMR Spectroscopy Utrecht, The Netherlands

Spectrometer	Sample state	Cryogenic probes available	Probes available	Channels available
900 MHz (Bruker Avance II)	Solution	5 mm TCI cryo HCN Z-GRD	5 mm TXI HCN XYZ-GRD 5 mm TXI HCP XYZ-GRD	Three + ^{2}H
750 MHz (Bruker Avance II)	Solution		5 mm TXI HCN XYZ-GRD 5 mm QXI HPCN Z-GRD 8 mm TXI HCN Z-GRD	Three + ^{2}H
700 MHz (Bruker Avance II)	Solution		5 mm TXI HCN Z-GRD	Three + ^{2}H
600 MHz (Bruker Avance DRX)	Solution	5 mm TCI cryo HCN Z-GRD	5 mm TXI HCN Z-GRD 5 mm BBI Z-GRD 5 mm QNI HCPN Z-GRD	Three + ^{2}H
600 MHz (Bruker Avance DRX)	Solution		5mm TXI HCN Z-GRD 5mm BBI Z-GRD 8mm TXI HCN Z-GRD 2.5 mm TXI HCN Z-GRD	Three + ^{2}H
500 MHz (Bruker Avance II)	Solution		5mm TXI HCN Z-GRD 5mm BBI Z-GRD 5mm TXI CIDNP Z-GRD	Three
500 MHz (Bruker Avance DRX)	Solution		5 mm QXI HCN Z-GRD 5 mm QNI HP Z-GRD 5 mm BBI Z-GRD	Three
360 MHz (Bruker AMX)	Solution		5 mm BBI Z-GRD	Two
500 MHz (Bruker Avance II)	Solid state (Wide Bore)		7mm MAS CP BB(N-P/H) VTN 4mm MAS CP Triple(N-P/H) BB sol 7.5 man/pneumatic CP BB(N-P/H) sol VTN 10mm BBI(D/Ag-P/H) 5/8mm Diff30 BBO(D-P/H)	Three

UNI BHAM (HWB-NMR) - University of Birmingham CR UK Institute of Cancer Studies (UNI BHAM) Birmingham, UK

Spectrometer	Sample state	Cryogenic probes available	Probes available	Channels available
900 MHz Varian INOVA	Solution	HCN 5mm z-PFG cryogenic probe with enhanced ^{13}C and ^{1}H sensitivity	HCN 5mm z-PFG probe	Four
800 MHz Varian INOVA	Solution	HCN 5mm z-PFG cryogenic probe with enhanced ^{13}C and ^{1}H sensitivity		Four
600 MHz Varian INOVA	Solution		HCN 10mm z-PFG; HX 5mm z-PFG; HCN 5mm z-PFG; gHX nanoprobe; 5mm ID z-PFG probe	Three
600 MHz actively cooled actively shielded Varian Direct Drive (AS768 autosampler)	Solution	HCN 5mm PFG cryogenic probe with enhanced ^{13}C sensitivity	HCN 5mm z-PFG probe	Three
500 MHz actively shielded Bruker DRX AVANCE (BACS60 autosampler)	Solution	HCN 5mm z-PFG cryogenic probe	HCN 5mm z-PFG TXI probe; 5mm 1H probe; 5mm $^{2}H/^{1}H$/BB probe; 5mm $^{2}H/^{1}H$/BB/^{13}C probe	Four
500 MHz actively shielded Varian Unity+	Solution		HCN 5mm z-PFG probe; Auto-X 5mm dual broadband probe ^{1}H-$^{19}F/^{15}N$-^{31}P; 5mm BB probe	Three
Hypersense system for dynamic nuclear polarization experiments		solid state metabolomics with dissolution device connected to Varian 500	^{13}C internal probe	N/A
funded, to be installed this year: 600MHz metabolomics station (NMR&MS)	solution	2 cryogenic probes for different sample volumes		Four

CNRS (RALF-NMR) - Centre National de la Recherche Scientifique, France: (a) UMR 5182 CNRS/Ecole Normale Supérieure de Lyon "Laboratoire de Chimie"; (b) UMR 5075 CNRS/Commissariat à l'Energie Atomique/Université Joseph Fourier, Grenoble

Spectrometer	**Sample state**	**Cryogenic probes available**	**Probes available**	**Channels available**
900 MHz	Solid-state/solution		4 mm HR-MAS Z-GRD ^{1}H, X 3.2 mm MAS ^{1}H, ^{13}C, ^{15}N 2.5 mm MAS ^{1}H, X (^{13}C to ^{31}P) 1.3 mm ^{1}H, X 5 mm BBO 5 mm TXI Z-GRD	Three +^{2}H
800 MHz	Solution	5 mm ^{1}H/^{13}C/^{15}N/^{31}P	-	Three +^{2}H
700 MHz	Solid-state/solution		4 mm HR-MAS ^{1}H, X 4 mm MAS ^{1}H, X (^{33}S to ^{31}P) 3.2 mm MAS ^{1}H, ^{13}C, ^{15}N 2.5 mm MAS ^{1}H, X (^{33}S to ^{31}P) 5 mm BBO 5 mm TXI Z-GRD	Four + ^{2}H
600 MHz	Solution	5 mm ^{1}H/^{13}C/^{15}N/^{31}P	-	Three +^{2}H
500 MHz	Solid-state (Wide Bore)		4 mm MAS ^{1}H, X, Y 4 mm MAS ^{1}H, X 2.5 mm MAS ^{1}H, X 2.5 mm MAS ^{1}H 7 mm MAS ^{1}H, X 4 mm HR-MAS Z-GRD ^{1}H, X	Three

Footnote:
Z-GRD, shielded gradient coil along Z axis