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Magnetic Resonance Microscopy Approaches to Molecular Imaging: Sensitivity vs. Specificity

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Abstract Magnetic resonance imaging (MRI) has become a staple of diagnostic radiology. Despite its diagnostic utility the resolving power of typical clinical MRI instruments is only on the order of 1 mm. This has led to the development of magnetic resonance microscopy (MRM), which employs the same physical imaging principals used in MRI, but with instrumentation designed to resolve structural details down to the level of 10–100 microns in samples ranging from less than 1mm to several centimeters in size. Until recently, major advancements in MRM have focused on hardware and software developments allowing the detection of radio-frequency signals originating from very small volume elements within the sample. Such high-resolution images have facilitated the early detection of diseased tissue by focusing on sub-millimeter structural changes induced in the tissue. To sensitize the MRM technique to pathologic tissue changes, investigators have developed techniques, such as chemical shift imaging to detect pre-cancerous changes in tissue metabolism and MR relaxometry to detect changes in tissue composition during the earliest stages of degeneration for diseases such as osteoarthritis or multiple sclerosis. However, such non-specific measurements can only serve as surrogate measures of disease progression and potential measures of treatment efficacy. As disease diagnosis moves from the anatomic to the molecular stage, scientists will require imaging techniques that can detect molecular events deep inside the human body. To meet this goal, MR scientists are working to improve imaging resolutions in vivo and they are developing molecular probes that can dramatically amplify the MR signal in response to specific and highly localized molecular events. This article will identify current trends in the MRM field aimed at meeting the challenges imposed by molecular imaging and areas for future development in this highly promising imaging field. J. Cell. Biochem. Suppl. 39: 147–153, 2002. © 2002 Wiley-Liss, Inc.

Key words: magnetic resonance microscopy; contrast agents; molecular imaging; molecular amplifiers; reporter molecules

BACKGROUND

In magnetic resonance microscopy (MRM), an image is constructed from spatially encoded radio-frequency (RF) radiation emitted by an object after the application of RF radiation at the appropriate resonant frequency. The source of the emitted signal is not from the applied RF radiation but from magnetic energy stored by the system. Magnetic energy can be stored by nuclei with non-zero spin-angular momentum. In biological imaging, protons (mostly from water) are the most common source of signal because they are abundant in tissues and they have a high magnetic receptivity, second only to tritium.

MR active nuclei, such as protons, in the presence of an external magnetic field behave like small magnetic dipoles (bar magnets) and store energy by distributing between a high energy (or anti-parallel) state where the magnetic dipoles are aligned against the external magnetic field and a low energy (or parallel) state where the magnetic dipoles are aligned with the external magnetic field. This distribution between parallel and anti-parallel energy

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states is governed by the classic Boltzmann equation. If one considers a large population of nuclei in an external magnetic field, the small excess of nuclei aligned with the field will produce a net macroscopic vector aligned along the main magnetic field direction. The magnitude of this vector is dependent on the strength of the external magnetic field, the sample temperature, and the magnetic dipole moment of the nucleus. The magnetic dipoles are not stationary in the field but are observed to precess about the axis of the external magnetic field (z-axis) at a resonant frequency, known as the Larmor frequency. This resonant frequency is the product of the magnetogyric ratio ($\gamma_H = 26.75 \times 10^7$ rad s^{-1} T^{-1} for protons) of the nucleus and the external magnetic field strength.

To detect the magnetic energy stored in a spin system, an external source of energy must be supplied to induce transitions between the two energy states and, accordingly, must exactly match the energy separation between the parallel and antiparallel energy states. The required energy is supplied in the form of electromagnetic radiation in the radio-frequency range of the spectrum. When the sample is exposed to RF energy at the Larmor frequency, the net magnetization vector rotates away from the z-axis and toward the x-y plane, oriented perpendicular to the direction of the external magnetic field. The resulting transverse component of the magnetization vector, which precesses at the Larmor frequency, induces an observable voltage in a sensitive coil placed in close proximity of the sample.

The detected signal has a resonant frequency, which is a function of the magnetogyric ratio and the local magnetic field experienced at the nucleus. Orbiting electrons act to reduce the effective field at the nucleus, resulting in a resonant frequency that is characteristic of the local chemical environment. This phenomenon is the basis of routine chemical analysis. The intensity of the signal is proportional to the total number of observed nuclei present in the sample. Finally, the rate at which the MR signal decays with time following excitation is characterized by two independent relaxation times T1 and T2. The T1 (or longitudinal) relaxation time measures the rate at which the spin system returns to the equilibrium distribution between parallel and anti-parallel energy states. This process is accelerated if the spin system can lose packets of energy to energy-requiring processes in the sample that occur at the Larmor frequency. The T2 (or transverse) relaxation time describes the rate at which energy is lost by irreversible, entropic processes within the spin system. This process is accelerated by magnetic field perturbations. Field perturbations can be very large if the magnetic dipoles are relatively fixed in orientation and position.

To spatially encode the magnetic resonance signal, a linearly increasing magnetic field gradient is superimposed on the constant, but much stronger, external magnetic field. Accordingly, nuclei at different spatial locations within the sample will experience different magnetic field strengths and thus different resonant frequencies. Thus, when the MR signal is subjected to Fourier analysis, the spectrum of resonant frequencies will reflect the spatial distribution of nuclei along the magnetic gradient direction. This is the basis of image generation.

An overview of the MR technique in terms of nuclear polarization, RF excitation, signal detection, and spatial encoding is summarized in Figure 1. For in depth coverage of the concepts introduced above, the reader is referred to two excellent books on the subject [Morris, 1986; Callaghan, 1991].

STRATEGIES TO IMPROVE SENSITIVITY

The fractional population difference between parallel and antiparallel spin states for protons in a 10 Tesla (100,000 gauss) magnet at room temperature is about 1 proton for every 100,000. With an inductive detection scheme, large numbers of spins are required to produce a detectable signal, making this approach unsuitable for single molecule studies. To improve the sensitivity of the MR technique, a number of strategies might be employed to improve the magnitude of the induced nuclear magnetism. One option is to increase the strength of the external magnetic field. Currently, ultra-high field magnets are commissioned to study the low levels of proteins expressed by cells. The maximum available field strength to date, however, is limited by magnetic saturation levels of superconducting metal alloys. Nuclear polarization can also be improved by lowering the sample temperature. However, this is not a viable approach for many biological applications. Another option is to establish a non-equilibrium polarization state in the spin population

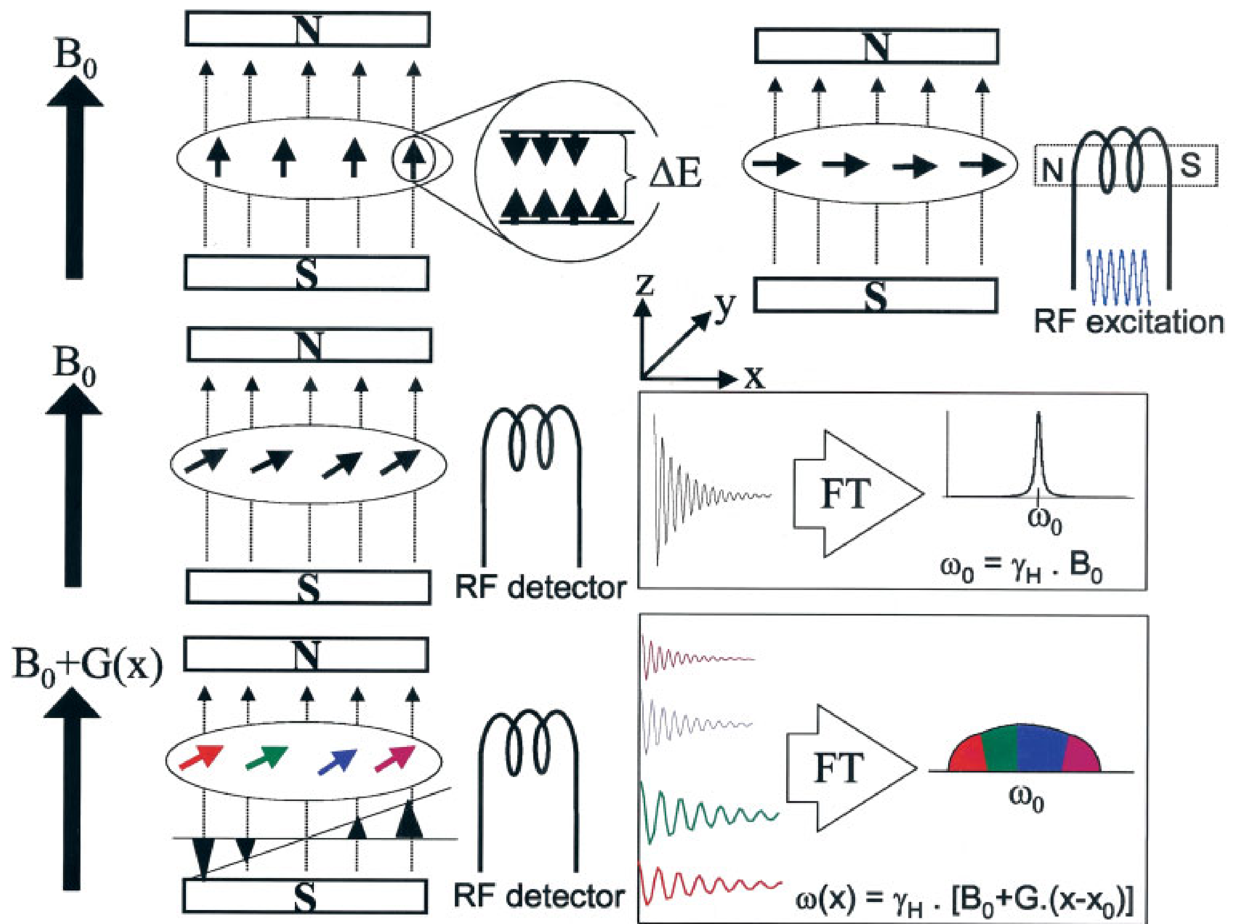

Fig. 1. In the presence of an external magnetic field (B_0), protons distribute between two energy states. Upon the application of a radio-frequency pulse at the Larmor frequency, all magnetic vectors rotate into the x-y plane. After RF excitation, the precessing magnetic vector induces a current in a RF detector. If all positions experience the same magnetic field strength, then the MR signal on Fourier analysis will have a single resonant frequency (ω_0) given by the Larmor equation. If a magnetic field gradient is applied along the x-axis, the protons on the left will experience a lower magnetic field than the protons on the right. This results in a distribution of MR signals in frequency space that matches the proton distribution along the x-axis.

with optical pumping. This approach has been employed to hyperpolarize xenon for lung imaging experiments [Song et al., 1999]. It is used mostly to excite noble gases because they remain hyperpolarized in the time it takes to deliver and image the gas.

An alternative approach for improving sensitivity is to improve signal reception. This has been achieved with the development of microcoils. Such coils have been used to study single cells or protein solutions in volumes comparable to cellular volumes [Lacey et al., 1999]. Significant improvements in sensitivity can also be achieved by reducing the thermal noise inherent in the detection system with the aid of cryocooled probes and preamplifiers. Another option, which has recently become available, is the development of parallel imaging techniques [Madore and Pelc, 2001]. Essentially, images are acquired in parallel rather than sequentially in time, reducing the total imaging time as well as improving signal reception because of the proximity of the RF coil array to the sample. Instead of inductive detection, the induced nuclear magnetism can be detected mechanically, using an atomic force microscope with a magnetic tip [Rugar et al., 1994]. This mechanical detection system has the potential to achieve atomic resolution, but it has yet to be applied to a biological system.

High field strength magnets and smaller RF probes have allowed for the detection of RF signals from small volume elements, and the use of very large magnetic field gradients has

allowed images with high spatial resolution to be generated. However, in conventional MRM, image resolution is limited to the mean displacement of water during the acquisition time, typically 10 microns at room temperature [Callaghan and Eccles, 1988]. For systems with short-range order, the diffusion behaviour of resident water molecules might be used to obtain size and size distribution information about the internal structures of the system [Callaghan et al., 1991]. This approach, called q-space imaging or MR diffusion-diffraction, has been used to obtain estimates of cellular dimensions in suspension cultures [Torres et al., 1998]. It exploits repetitive features or short-range order in the system to achieve sub-micron resolution. Spatially encoding the MR-diffraction pattern might be used to achieve subcellular resolution.

Image resolution might also be improved with super-resolution algorithms. Basically, subpixel shifted low-resolution images can be used to over-sample the image space and to obtain high-resolution images with improved signal to noise [Greenspan et al., 2002]. Another strategy is to over-sample in the time domain and take advantage of the eye's ability to do visual integration. Thus, low-resolution images, played at speeds faster than the time it takes for the eye to process the data, get averaged together resulting in a virtual improvement in image quality. Temporal imaging also exploits the eye's sensitivity to dynamic changes. Another approach is to use *a priori* geometric information about an image to estimate the true image and the blur from a low-resolution image. This difficult image analysis problem, described as blind deconvolution, has been used for image restoration in other applications but it has not been applied to low-resolution MRM images.

STRATEGIES TO IMPROVE SPECIFICITY

The MR technique, without any spatial encoding, is essentially a molecular technique. It can elucidate the structure of proteins in solution and when coupled with multivariate techniques it can be used for biochemical profiling of biofluids [Nicholson et al., 2002]. One of the biggest challenges in MR is the ability to study large molecular weight proteins within the cytoplasmic milieu. Even with advances in site-directed mutagenesis it is extremely difficult to resolve proteins with molecular weights in excess of 20 kDa. Furthermore, the detection of a specific protein in the presence of other proteins within the cell is a daunting task, even with the most sophisticated pattern recognition tools available. This has led to a new breed of reporter molecules or molecular amplifiers, which contain a magnetic label, instead of a fluorochrome, at one end and a receptor ligand or antibody at the other end. Unique protein molecules are not detected directly but indirectly with the help of a molecular amplifier, which dramatically changes the relaxation properties of surrounding water molecules when bound to the protein target, thereby making the MR technique infinitely more sensitive to very low protein concentrations. This approach has been used successfully to detect unique proteins within complex and often turbid mixtures of cell products without further purification [Perez et al., 2002].

Molecular amplifiers can be divided into two groups, T1 or T2 contrast agents. T1 agents act to reduce the T1 relaxation time of surrounding water molecules by providing at least one coordination site for water molecules to interact with the unpaired electrons of a paramagnetic ion or free-radical molecule. This effectively increases the signal from water molecules in the same voxel on T1-weighted images, which is especially useful in low signal-to-noise applications. T2 agents act to change the local magnetic field homogeneity. This is typically accomplished with superparamagentic nanoparticles. They produce areas of reduced signal intensity because of enhanced T2 relaxation processes and are best suited to high signal-to-noise applications.

MR contrast agents greatly improve the sensitivity and specificity of the MR technique. With the added capability of spatial encoding, MRM might be applied to lab-on-a-chip type studies. The advantage of the MR technique over traditional optical detection schemes is that there is very little contamination from other molecular species because there are few naturally occurring magnetic contaminants and there is little need for protein purification because probe molecules can be detected in systems that are turbid or optically opaque. This is a significant advance considering that current molecular imaging strategies can only be performed in monolayer cultures or in developmental models that are optically transparent.

EVOLUTION OF A MR-BASED MOLECULAR DIAGNOSTIC TOOL

In 1971, Damadian excited the medical world when he published his article entitled "Tumor detection by nuclear magnetic resonance" in which he reported distinct differences in the proton T1 relaxation times of normal and neoplastic tissues [Damadian, 1971]. The possibility of making MR measurements in vivo motivated scientists to assess the value of MR relaxation parameters for characterizing pathologic and normal tissues. This led to the expectation that such measurements could be used in the early detection of cancer and for monitoring therapeutic treatments. This gave scientists the impetus to develop the clinical MRI scanner. MR relaxometry, however, did not lead to significant improvements in the early detection of cancer. It did, however, yield valuable correlations between MR measurable parameters and important tissue specific biochemical parameters, which form the basis of early detection schemes for diseases such as osteoarthritis and multiple sclerosis. At the same time, chemical shift imaging has yielded important metabolic markers for the early detection of cancerous lesions, and the use of compartmental contrast agents for studying changes in blood flow, tumor angiogenesis, and vessel wall permeability has improved the sensitivity of the MR technique to sub-clinical tumors. With the ability of MRM to acquire images with sub-millimeter resolution, much of the work required to improve disease diagnosis in humans can be developed in animal models. It is expected that animal imaging studies will greatly accelerate the development of enabling technology for disease assessment in humans.

As scientists begin to understand the cellular basis for disease several groups, notably with expertise in fluorescence microscopy, have developed molecular amplifiers that allow for the detection of unique cell populations in the MRM image. The earliest experiments involved micro-injecting a single cell with a membrane-impermeable contrast agent that would remain within the originally labeled cell and its descendants [Jacobs and Fraser, 1994]. Alternatively, cell surfaces have been labeled with monoclonal antibody-conjugated MR contrast agent [Nunn et al., 1997]. While this approach has been used to detect tumors in mice, high levels of the contrast agent end up in the liver and spleen. In any event, scientists have demonstrated that low-resolution MR images with an appropriate magnetic label can be used to track unique cell populations in vivo. For example, scientists can now label populations of macrophages with magnetic nanoparticles, internalized by endocytosis, to study various inflammatory diseases, such as atherosclerosis and autoimmune diseases [Dousset et al., 1999]. To improve the labeling efficiency, magnetic nanoparticles can be derivatized with the appropriate conjugate for transport via a number of cell-surface transporters [Lewin et al., 2000]. Conversely, cells might be transfected to overexpress cell surface receptors for shuttling increased amounts of magnetic label into the cell [Lewin et al., 2000].

The challenge at the moment is to sensitize the MR signal to molecular level changes. To do this, scientists have exploited at least two marker genes. The first marker gene, alluded to earlier, encodes for cell-surface receptors and has been used to increase the number of cell surface receptors needed for internalizing magnetic nanoparticles [Weissleder et al., 2000]. The second marker gene encodes for intracellular enzymes and has been used on conjunction with an activatable reporter probe to map endogenous levels of protease activity [Louie et al., 2000]. Both strategies have been used to map the successful transfection and gene expression levels of cells in vivo. How this will translate into human studies is yet to be seen. It is too early to speculate how scientists intend to overcome the many barriers to the delivery of molecular amplifiers. We can, however, speculate that cells used for cell-based therapies might provide the requisite vehicle for the transport of molecular amplifiers. This could potentially revolutionize the monitoring of cell-based therapies such as gene therapy, stem-cell therapy, and tissue engineering.

A timeline for the evolution of a MR-based molecular diagnostic tool is presented in Figure 2. This list is by no means extensive and is meant to highlight key discoveries, which make it possible to conceive of MR approaches to molecular imaging.

CONCLUSIONS

Recent advances in cell biology and genomics have created a voracious need for techniques that can analyze the hundreds of thousands of

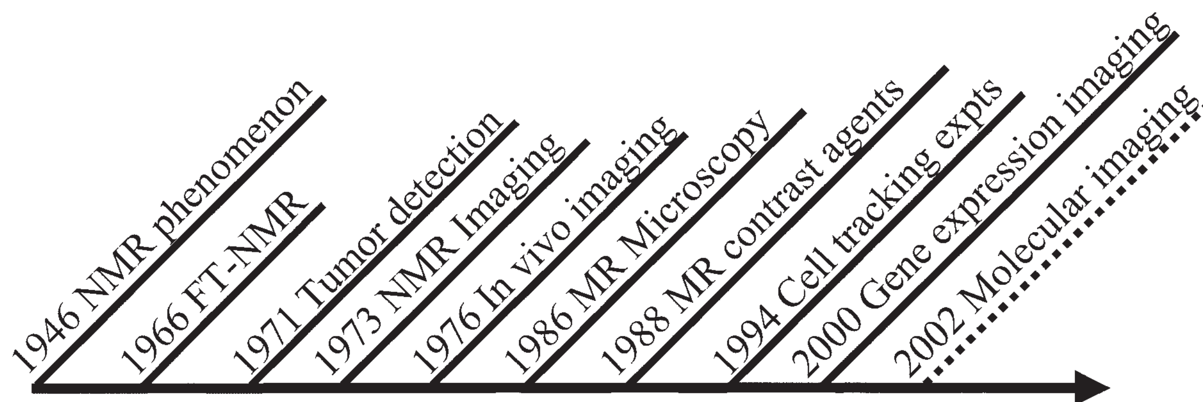

Fig. 2. The nuclear magnetic resonance (NMR) phenomenon was discovered simultaneously and independently in 1946 by groups headed by Block and by Purcell. In 1966, Ernst and Anderson demonstrated the multiplexing advantage of Fourier transform NMR. Damadian, in 1971, alluded to the medical diagnostic possibilities of NMR, and in 1973 Lauterbur published the first two-dimensional NMR image. The first human MR image was reported 1976 and the first MR image of a single cell was reported 1986. In 1988, the FDA approved the first MR contrast agent (Gd DTPA) for clinical use and the earliest cell tracking experiments were reported by a number of groups in 1994. Mapping gene expression with MR was reported in 2000. Finally, in 2002 the prospect of a MR-based molecular diagnostic tool has been formally recognized.

different proteins that are expressed in the human body. Magnetic resonance, without spatial encoding, has already been recruited to study protein expression levels of cells in vitro. The current interest in proteomics is expected to drive the development of higher field magnets and better detection systems. At the same time, molecular amplifiers will continue to be developed to improve the sensitivity of the MR technique to low protein concentrations. The advantage of a MR-based technique is that the system does not have to be optically transparent and signals can be spatially encoded so that more than one sample can be interrogated at one time. Such a detection scheme might be applied to high-throughput assays. As the tissue or cell arrays gets smaller, we might have to resort to a mechanical detection system in which the induced nuclear magnetism is detected with an atomic force microscope. This approach has the potential to achieve atomic scale resolution with good chemical specificity.

With regard to MRM, scientists will continue to grapple with parallel imaging strategies, super-resolution algorithms, and q-space imaging to improve upon current limits in resolution. While these approaches might get us closer to true microscopic imaging, they do not address the more important question of specificity. To detect molecular events deep inside the human body, we need to develop better amplification strategies. Currently, much of the work to date has borrowed ideas and concepts developed for fluorescent microscopy, where it is sufficient to use a single fluorescent label to detect a single molecular event. This amplification strategy will not be sufficient when trying to detect the spatial location of a molecular event in three dimensions. Molecular amplifiers need to be designed such that a single binding event can localize thousands of paramagnetic ions rather than 1 or 2 ions as seen with today's MR reporter molecules [Moats et al., 1997; Li et al., 1999]. Another idea is to use high concentrations of other MR active nuclei to eliminate background signals. Once the binding event is detected, a proton image can be acquired to locate the event. To enhance the sensitivity of the MR experiment to magnetic nanoparticles, smaller particle sizes and higher magnetic fields are warranted.

However, the success of any molecular amplification strategy is limited by the delivery and targeting of molecular probes. One way around the delivery problem is to use therapeutic cell populations to transport the molecular probe or receptor transgene to the target site. The long-term benefit will be that scientists can then monitor the cell-based therapy in vivo. Alternatively, biodegradable polymer scaffolds

loaded with plasmid DNA might be used to transfect cells with marker genes required for detection by MRM [Shea et al., 1999].

In conclusion, the development of MRM approaches to molecular imaging will allow for the study of molecular events in more complex organisms with sub-millimeter resolution. This is a far cry from other techniques which might be either infinitely more sensitive but which do not provide good spatial localization, or which do not have good depth penetration and are therefore limited to systems that are optically transparent. To meet the stringent requirement of molecular imaging, we will require improvements in sensitivity for better spatial localization and improvements in amplification strategies to sensitize the MR technique to molecular level events in vivo.

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