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Active Staining of Mouse Embryos for Magnetic Resonance Microscopy

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Abstract

Magnetic resonance histology (MRH) has found considerable application in structural phenotyping in the mouse embryo. MRH employs the same fundamental principles as clinical MRI, albeit with spatial resolution up to six orders of magnitude higher than that in clinical studies. Critical to obtaining this enormous gain in resolution is the need to enhance the weak signal from these microscopic voxels. This has been accomplished through the use of active staining, a method to simultaneously fix the embryonic/fetal tissues, while reducing the spin lattice relaxation time (T1). We describe here the methods that allow one to balance the fixation, which reduces the NMR signal, with the enhancement of signal derived from the reduction in T1. Methods are included to cover the ranges of embryonic specimens from E12.5 through E19.5.

Keywords

Magnetic resonance microscopy; embryos; active staining

1 Introduction

Histology as defined by Webster's dictionary is "the branch of biology concerned with the microscopic structure of tissue." The vast majority of the histology studies today, and the focus of much of this book, employ conventional optical or electron microscopes. Magnetic resonance histology (MRH) was first suggested by Johnson, et al. in the early 1990s (1,2). MRH differs from conventional optical methods in four major ways: 1) MRH is non-destructive: there is no physical sectioning required such that the tissue under study remains physically intact; 2) The contrast in the MR histology images is dependent on the protons (mostly water) in the tissue and how those protons are bound. This, in turn, provides a rich bounty of contrast mechanisms to differentiate structures and pathology based on the same parameters that have made MRI so successful in the clinical domain—such as spin lattice relaxation (T1), spin spin relaxation (T2), diffusion, and proton density. The underlying physics of these "proton stains" (2) is beyond the scope of this chapter. The interested reader is referred to several excellent texts (3,4,5). 3) MRH provides three-dimensional images; and 4) MRH is inherently digital.

MRH is based on the same physical principles as clinical MR imaging (MRI). However, MRH differs from MRI in several respects. MRI studies of post-mortem specimens have been performed using clinical systems (6). But, clinical systems are typically limited to spatial

resolution on the order of 1 mm. Systems designed for MRH provide spatial resolution down to 10 μm . Since MRI and MRH are tomographic (three-dimensional) imaging systems, spatial resolution is most appropriately stated in terms of the voxel volumes. Thus, the dedicated systems, with 10 μm^3 voxels (voxel volume = 1 pL) are encoding the signal from voxels that are 1,000,000X smaller than the clinical systems at 1 mm^3 (voxel volume 1 μL). To achieve this increase in spatial resolution, systems designed for MRH differ significantly from clinical MRI systems. The first major difference is in the magnetic gradient coils. Spatial encoding in MRI/MRH is achieved through the application of a magnetic field gradient (7). In order to achieve the higher resolution, MR microscopes use much stronger gradients. The typical MRI system achieves gradients of $\sim 50 \text{ mT/m}$. The gradients on an MR microscope can be 60X greater (3000 mT/m). Technical constraints limit the volume over which such gradients can be sustained, so the magnets used for these studies are much smaller bore (60–120 mm) than the 1 m bore of a clinical magnet. This, of course, limits the size of specimen that can be studied.

Since the voxels are 1 million-times smaller, the signal is 1 million-times weaker. Thus, a major focus in MR histology is increasing the sensitivity. This is achieved in three ways. First, MRH systems use much stronger magnets: clinical MRI systems operate at 0.5T–3.0T, while MRH systems work at 7.0T–22.0T. Second, since the MRI/MRH signal is a radiofrequency (rf) signal that is captured by the rf probe that holds the specimen, careful design of the probe is essential to optimize the sensitivity. But, this also (like the gradient coil) limits the size of the specimen. Smaller radiofrequency coils provide greater sensitivity required for higher spatial resolution. The third approach to enhancing sensitivity is the use of active staining (8), which is the focus of this chapter. The signal in MR histology is derived from protons (usually water) in the tissue. The nuclear magnetic resonance (NMR) phenomenon, upon which MRI/MRH is based, exploits the interaction of a radiofrequency pulse to excite these protons. Once excited, the protons return a signal, the strength of which is dependent on intrinsic factors in the tissues and extrinsic variables that are set in the scan protocol (3). For example, for a T1-weighted sequence, the signal S from a tissue is given by Equation 1:

$$S_i = PD_i(1 - e^{-TR/T1_i}) \quad [1]$$

where PD_i , the proton density in tissue i , and $T1_i$, the spin lattice relaxation for tissue i , are intrinsic for that tissue, and TR is the extrinsic parameter that one sets in the scanning sequence. Fig. 1 shows a graph of the signal for two tissues with differing spin lattice relaxation times. Tissue A (dotted line) has a shorter T1 than tissue B (solid line). At $TR1$, the signal from tissue A is much greater than it is from tissue B. Active staining is the process of radically reducing spin lattice relaxation time through the induction of a chemical agent.

Fig. 2 shows both the impact of changing the extrinsic variable (TR) and the intrinsic tissue parameters ($T1$). Figure 2(a–d) shows images of a formalin-fixed mouse brain acquired with TR of 20, 40, 80, and 160 ms. Figure 2e–f shows an identical series for a mouse brain that has been actively stained (9). These images are acquired at very low spatial resolution to allow quick comparison of the consequence of active staining. The signal enhancement from active staining is 8.4X at $TR = 40 \text{ ms}$. The benefits of active staining are clear: under optimized conditions, one can realize gain in signal-to-noise, gain in spatial resolution, and gain in contrast. Here, we describe the methods we employ with specific focus on the mouse embryo.

2 Materials

2.1. Preparation of the fixing-staining solution

1. Bouin's solution (LabChem Inc., Pittsburgh, PA, USA) (*see* Note 1).

2. Gadoteridol (ProHance®, Bracco Diagnostics Inc., Princeton, NJ, USA), 0.5 M (*see* Note 2).
3. Eppendorf tubes for embryos of CRL $\sim \leq 5$ mm.
4. 15-mL jars for embryos of ~ 5 mm $\leq$ crown-rump length (CRL) ≤ 17 mm (*see* Note 3).
5. 30-mL containers for fetuses of CRL $\sim > 17$ mm.

2.2. Preparation of the storage solution

1. Phosphate buffered saline (PBS).
2. Gadoteridol (ProHance®, Bracco Diagnostics Inc., Princeton, NJ, USA), 0.5 M.
3. Eppendorf tubes for embryos of CRL $\sim \leq 5$ mm.
4. 15-mL jars for embryos of ~ 5 mm $\leq$ CRL ≤ 17 mm (*see* Note 3).
5. 30-mL containers for fetuses of CRL $\sim > 17$ mm.

3 Methods

3.1. Immersion fixation-staining of E9.5–E18.5 (Fig. 3)

1. Dilute some ProHance in Bouin's at 1:20 (v:v, i.e. 25 mM) and prepare 15 mL–30 mL per embryo/fetus, depending on the size (*see* Note 1,^{2,4}).
2. After hysterectomy of the anesthetized pregnant mouse, dissect out the first embryo/fetus in a Petri dish filled with ice-cold saline and cut the placenta off if you do not need it. Drop the embryo/fetus in a jar with the fixing-staining solution at room temperature. See Table 1 for immersion durations (*see* Note 5,^{6,7}).

3.2. Immersion fixation-staining of E19.5 (Fig. 4)

Late-stage fetuses are viable, so care should be taken not to fix them if their heart is still beating. The fetuses can be intraperitoneally (i.p.) injected with diluted anesthetics at lethal dose prior to fixation.

Another issue arises from the impermeable skin that does not allow the reagents to penetrate. This can be overcome by several injections of the fixing-staining solution prior to a 24-hour immersion (30-mL container): i.p. in the abdomen to help penetration in the lower body parts, or subcutaneous in the neck to help penetration in the head (*see* Note 5,^{6,7}).

¹Other fixative agents, such as formalin, can also be used. We have chosen Bouin's because the picric acid aids the diffusion of the contrast agent into the tissues. Other fixatives will permeate the tissue at different rates, so the immersion times will need to be adjusted. In addition, the cross-linking that causes reduction of T2 may be different, so the results may vary from those shown here.

²Other paramagnetic contrast agents, such as gadopentetate dimeglumine (Magnevist®, Bayer Schering Pharma, Germany) or gadoterate meglumine (Dotarem®, Guerbet, France) can also be used, though one needs to be cognizant of potential chemical reactions. For example, MnCl₂, a potential, inexpensive stain, may precipitate in some fixatives.

³For the fixation solution, it is generally recommended to use 15 to 20X the volume of the specimen, but 15 mL is sufficient for most stages.

⁴MR is generally sensitive to water protons. Fixatives that remove all the water (e.g. alcohol), remove the source of the MR signal. While there are protons in alcohol that do yield an NMR signal, they are not chemically equivalent and this can lead to severe artifacts in the MR images.

⁵This method can be extended to rat embryos/fetuses/pups and to all mouse strains, as well.

⁶This method can also be used for fixing-staining other tissue types of comparable sizes (e.g. mouse brains). But where possible, perfusion fixation provides better results (10).

⁷Active staining has been used for much larger specimens, e.g. post-natal mice, whole adult mice, and isolated organs (8). While immersion fixation is possible, larger specimens will require much longer immersion times and as with all fixation, the quality of the fixation (and staining) can be improved with direct perfusion.

3.3. Long-term storage of fixed-stained specimens (see Note 8)

The staining process is designed to reduce the spin lattice relaxation time (T₁), thereby increasing the MR signal. This is accomplished by the gradual diffusion of the contrast agent into the tissues. But, the cross-linking that accompanies the fixation has detrimental impact on the MR signal that arises from reduction of a second critical MR parameter, the spin spin relaxation time, T₂ (10). Fixation beyond the time required for the contrast agent to diffuse into the tissue is not recommended. Fig. 5 shows a comparison between two fetuses fixed for 3.5 and 24 hours, respectively. The fixation and staining can be stabilized after the appropriate fixation time by immersion in a PBS/contrast agent solution with a low concentration of contrast agent (to avoid over-staining of the tissues). A dilution of 1:200 ProHance:PBS should be used and the specimens should be stored at 4°C. Specimens can be stored for several weeks in this solution.

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⁸This method is compatible with post-histological analysis of the fixed tissues (Fig. 6). Paramagnetic contrast agents have no known interferences with most routine (e.g. H&E, Nissl) histological staining.

Fig. 1.

Signal intensity as a function of TR for two tissues with different T1. The solid line shows the signal from a tissue with long T1. Active staining reduces the T1 yielding the recovery curve shown by the dotted line. With active staining, one can use much shorter TR and still recover the majority of the signal (reproduced from ref [10] with permission of the Journal of Magnetic Resonance Imaging).

Fig. 2.
a–d) MR microscopy images from a formalin-fixed mouse brain (at TR= 20, 40, 80, and 160 ms, respectively) show increasing signal with longer TR. e–f) MR microscopy images of an actively stained mouse brain (at TR= 20, 40, 80, and 160 ms, respectively) show a much enhanced signal (reproduced from ref [9] with permission of NeuroImage).

Fig. 3.

Embryos from E10.5 to E18.5: (a) mid-coronal slices and (b) rendered volumes. The contrast across images is very similar and many small structures are clearly identifiable. These data are part of an extensive database of the developing mouse (11) available online at <http://www.civm.duhs.duke.edu/devatlas/index.html>.

Fig. 4.

E19.5 fetus prepared with i.p. injections of ProHance:Bouin's at 1:20 followed by an overnight immersion in the same mixture. High signal-to-noise-ratio (SNR) and contrast-to-noise-ratio (CNR) were achieved uniformly throughout the body with no significant tissue damage or image artifact. The arrows point to the sites of injection (neck and lower abdomen).

Fig. 5. Coronal slices from 3D acquisitions (3D spin warp, TR/TE = 100 msec/5.5 msec, FOV = $20 \times 10 \times 10$ mm, matrix size = $1024 \times 512 \times 512$) of E18.5 rat fetus immersed for (a) 3.5 hours and (b) 24 hours. Note the strong signal from the fluid in the ventricle indicating that the T1 has been reduced in both cases. But the extended immersion also reduces T2 (in b) resulting in much lower MR signal (1).

Fig. 6. Coronal slices of (a) MR histology and (b) conventional histology (hematoxylin and eosin stain) of the same E16.5 mouse fetus. The specimen was fixed for 4 hours and stored for 2 months prior to sectioning.

Table 1

Immersion durations as a function of the size of the specimen (± 0.5 mm) and reduced T1 values

Stage	E10.5	E11.5	E12.5	E13.5	E14.5	E15.5	E16.5	E17.5	E18.5
CRL (mm)	4	5	7.5	9.5	11	12	13	15	16.5
Immersion duration	10 min	10 min	30 min	30 min	1 h	2 h	4 h	9 h	24 h
T1 (ms)	35	53	40	80	53	87	85	70	50