Technique Notes

Methods and rationale for high-resolution magnetic resonance imaging (MRI) of *Drosophila*, using an 18.8 Tesla NMR spectrometer.

Null, Brian[1*]**, Corey W. Liu**[2]**, Maj Hedehus**[3]**, Steven Conolly**[4]**, and Ronald W. Davis**[1]. [1]Stanford Genome Technology Ctr/Bio-X Program; [2]Stanford Magnetic Resonance Laboratory; [3]Varian Inc, NMR Instruments; [4]U.C. Berkeley, Dept. of Bioengineering; *Corresponding author

Introduction

Magnetic resonance imaging (MRI) is proven as an important tool for the study of thick or opaque tissues in living organisms, and its application to the study of development and biomedicine in the smallest model organisms is an exciting frontier. NMR spectrometers, while not typically used for imaging, are capable of generating extremely high magnetic fields, up to approximately 20 Tesla, whereas the more familiar MRI devices used to image humans in the clinical setting operate at only about one Tesla. High field strength is especially important for tiny specimens like the fruit fly, to increase signal to noise ratio and NMR spectral resolution for quantitation of metabolites *in vivo*. The small sample dimensions of the NMR spectrometer are ideal for the study of *Drosophila* and other small model organisms. Further, the ongoing innovation of MR contrast agents which can act as *in vivo* indicators of physiological status such as calcium ion concentration and gene expression, combined with the robustness of *Drosophila* as a model organism with a diverse array of genetic tools and genomic data, would make *in vivo* imaging and spectroscopy a highly desirable technique for the study of *Drosophila*. Over the past several decades, tremendous advances have been made in the capabilities of magnetic resonance methods for imaging and spectroscopic measurement in human subjects. Such methods are increasingly being developed and utilized for small mammal studies; however, while adapting the methods and instrumentation used for human patients to the rodent models has been highly beneficial, it will always face limitations which can be easily overcome by working in the fly. And recent developments in magnetic resonance instrumentation and methods have greatly increased the feasibility of detailed *in-vivo* spectroscopic study of *Drosophila*. The methods described here are part of an ongoing effort to expand the capabilities of magnetic resonance methods and build on our prior genomic studies to aid the future development of new human disease models.

MRS, or magnetic resonance spectroscopy, is a special case of MRI, in which a sample can be analyzed to acquire NMR spectral data for discrete regions of tissue within the magnetic resonance image. MRS uses the excellent chemical specificity of the nuclear magnetic resonance technique to allow the non-invasive quantitation of certain metabolites, which can be correlated with structural and functional data. For example, MRS can easily identify the three phosphate groups of ATP, the energy buffer phosphocreatine, and inorganic phosphate, from which intracellular pH can be derived, allowing the bioenergetic status of tissues to be accurately assessed. When MRS is applied to an intact biological specimen, it begins to open the door to direct detection and measurement of the molecular physiology encoded in the biochemistry of a living organism.

For example, if spectroscopy of neurotransmitters can be accomplished in the living, developing fly, as it has been in the rat, and these data are coupled with the expression data of the

genes involved in neurotransmitter-mediated signal transduction and feedback systems, it will be a powerful tool. A synergistic combination of imaging and genomic tools stands to greatly accelerate research in *Drosophila* disease models and thus our understanding of the molecular-genetic nature of human diseases.

The ideal specimen for high resolution *in vivo* imaging and spectroscopic study must be hardy and unmoving, capable of remaining stationary for long periods of time while immersed in an oxygen-rich perfluorocarbon oil. Additionally, *in vivo* spectroscopy is most effective if the instrumentation is closely matched to the size of the specimen, and generates the highest possible magnetic field. The *Drosophila* pupa has particularly excellent properties as an MRI subject, in that it is immotile and also naturally capable of gluing itself to glass surfaces, where it remains for ~100 hrs (at 25°C) while undergoing massive developmental reorganization. The opacity of the pupa and adult phases have limited the study of these periods of the life cycle, as investigators have primarily used the light microscope as the instrument of choice.

Of course, light microscopy is indispensable, and in comparison to MRI, conventional imaging using light microscopy has great advantage in the ease of achieving real-time temporal resolution. However, there are obviously limits to the techniques of light microscopy, and there is great opportunity for MRI to be used as a highly complementary tool to existing methods. Light microscopy is limited to tissues that transmit light, and as resolution is increased, the field of view decreases, and the light intensity must be increased dramatically, so that as higher spatial resolutions are approached using methods like confocal microscopy, the intensity of light radiation can easily damage or kill a live specimen. Generally, research which requires detailed light microscopic studies of insect and vertebrate models is limited to dead or damaged specimens, requiring special dissection, chemical fixation, or other deleterious preparation, which can include partial dissection of a live specimen, to afford access of the light microscope to a tiny field of view within the specimen. Although the resolution of light microscopy is fundamentally only limited by the wavelength of light, in practice high resolution *in vivo* studies are severely limited by light requirements and diminishing field of view with increasing resolution, as well as deleterious and logistical challenges. While it may be straightforward to optically image a thin slice of tissue with 10 micron resolution or better, it is hard to imagine any practical method other than MRI for imaging the entire surface of a live animal, in addition to the full volume of its interior, at this resolution. This report describes methods we have employed to this end, imaging *Drosophila* in an NMR spectrometer, in an effort to ascertain the feasibility of MRI and MRS on live *Drosophila* at 18.8 T, with a field of view less than 5 mm. The coming challenge for this technique will be to improve methods and instrumentation for greater spatial and temporal resolution of imaging and spectroscopy *in vivo*.

Instrumentation, Methods, and Findings

Proton resonance imaging was performed at the Stanford Magnetic Resonance Laboratory on a Varian 800MHz NMR instrument with vertical bore magnet (Oxford Instruments), the Varian INOVA console and 5 mm inner diameter triple-resonance H {CN} triple-axis gradient NMR liquids probe. Specimens were immersed in a gas-permeable fluorocarbon oil, either "Halo 700" or "Halo 27" (Halocarbon, Inc), which are high molecular weight polymers of chlorotrifluoroethylene [PCTFE] used to prevent desiccation, improve magnetic susceptibility matching between the specimen and the surrounding medium, and decrease background hydrogen signal. Viability testing demonstrated flies were able to survive to adulthood (eclosion) in this oil while subjected to prolonged imaging protocols.
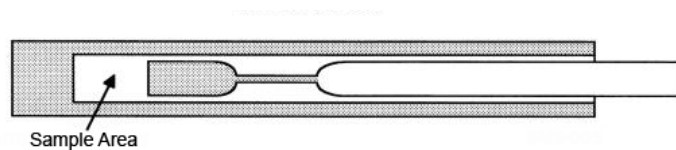
At Left: The 800MHz (18.8 Tesla) Varian NMR Spectrometer. Samples are loaded from the top, with the aid of a stepladder.

Imaging sequences utilized include spin-echo multi-slice, 3D spin echo, and 3D gradient echo. Experimental parameters have varied among experiments and are generally in the following ranges FOV: 2-5 mm, TR: 0.1-2.0 s, TE: 2-25 ms, matrix: 64x64x64 or 128x128x128, scan time ranges greatly, depending on the experiment, from 15 minutes to 12 hours. Pupal flies subjected to an intensive imaging regimen remain viable in this medium and develop to adulthood. Specimen mounting is fairly straightforward, without special oxygenation or anesthesia. Notably, internal movement and developmental speed can be slowed or accelerated by changing the temperature of the specimen using the variable temperature controller of the INOVA instrument console.

Specimen Preparation in NMR Sample Tubes:

Individual adult and pupal *Drosophila melanogaster*, as well as *D. bifurca*, and *D. aracatacas* (Tucson Stock Center #15085-1621.0 and #15040-1171.0) were imaged in 5 mm diameter NMR tubes, either in air or immersed in halocarbon oil. Numerous arrangements were devised to hold the specimen, the most streamlined of which as follows: Samples are placed in 5-10 mm long sections of glass capillary tube that have been flame-sealed at one end. Prepupae and pupae can be gently collected from culture vials and placed by hand, or, for minimal invasiveness, the 'wandering 3rd instar' larvae can be allowed to crawl inside these tubes and glue themselves to the inside as they form white prepupae. Prior to imaging, the tube is filled with halocarbon oil, just covering the pupa. These miniature sample tubes containing the specimen are then placed inside a 5 mm NMR tube and loaded into the spectrometer for imaging. For vertical alignment of the sample and chemical calibration, a special NMR tube was used which contains another smaller diameter tube within it to form two separate sections, concentrically aligned. With the sample placed in the inner section, it is vertically aligned along the long axis of the tube, and the outer section can be filled with 100% D_2O or similar reagent to allow for calibration of the NMR instrument. A benefit of vertical mounting of the specimen is that several samples can be vertically stacked in one tube and then imaged individually, by either moving the field of view via the software interface, or by physically moving the sample tube up or down within the instrument.
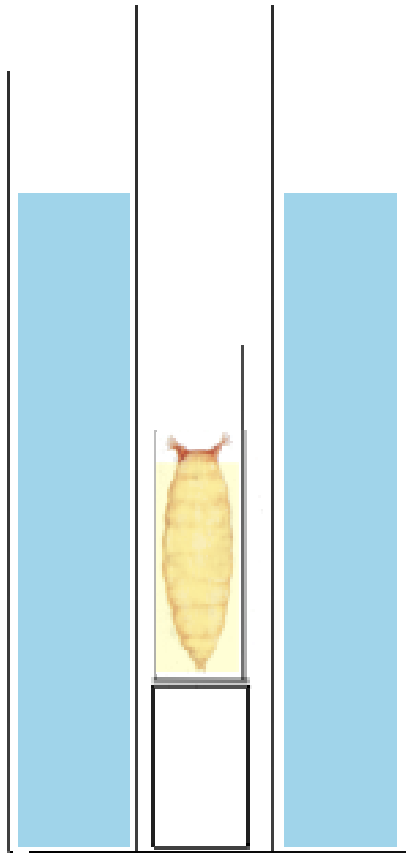
This concentric tube method was used for most imaging experiments; however, with the INOVA 800 system and the particular probe hardware used in this case, for higher resolution scans, it was sometimes preferable to mount the specimen in a transverse (horizontal) configuration. To achieve horizontal alignment of the sample, it can be placed in a 'shigemi' type NMR tube, which has a flat horizontal surface at its bottom, and a glass plunger which fits within the tube and holds the sample in place (Shigemi, Inc., sigma #Z54,334-9).

Sample Area

Shigemi tube diagram

Alternatively, a twisted pair of long glass fibers, monofilament fishing line, or similar mechanical support can be mounted inside a normal 5 mm diameter sample tube (Norell, Inc. #509-UP-8), holding the specimen very lightly like a pincer between the fibers (fabricated in-house). Similarly a glass capillary tube wrapped in spacers made from wrapped strips of Parafilm M (VWR #52858-000) may be mounted concentrically inside the NMR sample tube, the specimen attached at its terminus with double-stick tape, and immersed in halocarbon oil at the bottom of the tube; however, this method can create a syringe-like pressurizing effect, potentially causing issues with air bubbles and hydraulic complications which can be quite challenging, and so is best used as a 'quick and dirty' approach for machine calibration and so forth rather than real biological measurements.

        Mounting of adult flies can be accomplished by similar mounting methods as used for pupae, though additional care must be taken due to their greater fragility. When immersed in the halocarbon oil, the adult flies react similarly to anesthetization with CO_2 or ether: all movement ceases, and the fly remains in a characteristic life-like position, which is generally not the case for treatments that kill the fly directly. Larvae, when placed in halocarbon oil, appear unfazed and continue to move normally. Thus larvae will require further refinement of the specimen mounting technique for imaging. For larvae it might suffice to cool the sample enough to still the larva's movement (controllable from the INOVA interface), or administer a chemical anesthetic.

Schematic cross section of lowermost portion of the concentric two-chamber NMR tube with pupa mounted vertically and the outer chamber filled with 10% D_2O (dimensions and tolerances not drawn to scale). Exact tube used in no longer available, but a suggested replacement is WILMAD-LABGLASS products #517, 518, or 519-"COMPLETE".

Administration of MR Contrast Reagents (CR) to *Drosophila*:
        A gadolinium-based contrast agent ('Magnevist', Berlex Inc.) and a solution of manganese chloride were separately administered and demonstrated effective both by direct feeding and microinjection during larval, pupal, and adult phases of the life cycle, while retaining viability.
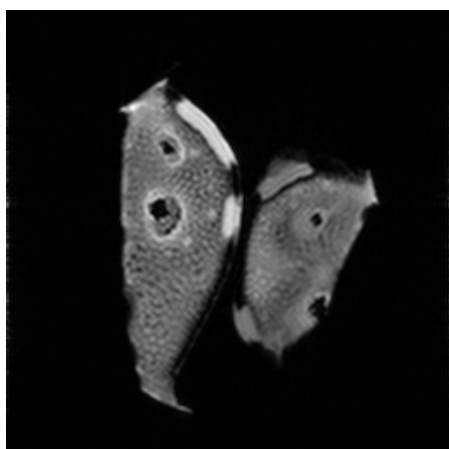
$MnCl_2$ in food:

Final Concentration	Effect
100 mM	Adults die
50 mM	Adults persist, but no offspring
20 mM	Adults persist, but no offspring

MnCl$_2$ Preparation: Starting with 1 Molar MnCl$_2$ solution (Sigma #M1787), a 100mM MnCl$_2$ food mixture was prepared by adding 1M solution to heated, molten *Drosophila* food medium and mixing well. 50 and 20 mM food was prepared by further dilution of the 100 mM medium.

Gadopentate Dimeglumine:

　　　Magnevist Injection (Berlex Inc.) is a 0.5 mol/L solution of 1-deoxy-1-(methylamino)-D-glucitol dihydrogen [N, N-bis[2-[bis(carboxymethyl)amino]ethyl]-glycinato-(5-)-] gadolinate(2-) (2:1), a paramagnetic contrast medium used diagnostically for clinical (human) MRI. Each mL of Magnevist Injection contains 469.01 mg gadopentetate dimeglumine, 0.99 mg meglumine, 0.40 mg diethylenetriamine pentaacetic acid and water for injection. The contrast enhancing effect of Magnevist is caused by the di-N-methylglucamine salt of gadopentetate (Gd-DTPA), the gadolinium complex of diethylenetriamine pentaacetic acid. With the appropriate imaging sequence (*e.g.* with T$_1$-weighted spin echo), the resultant shortening of the spin-lattice relaxation time of excited nuclei upon exposure to gadolinium allows an enhancement of signal intensity while reducing acquisition time. **As a preliminary control test of Magnevist in our system, we placed freshly severed sections of plant stem (*Apium graveolens*) into a solution of Magnevist with food dye, and a control solution without Magnevist, overnight. The food dye was observed to travel up the vascular tissues of the stem, and then excised slices of each were imaged side by side, across a series of relaxation times. The results show nicely how Magnevist enhances the signal in tissues:

Plant tissue demonstration of contrast agent: Magnevist-treated sample, on left side, shows brighter signal surrounding vascular tissues than in the non-treated sample, on the right. Spin-echo multi slice (SEMS) method, 256x256 grid, 19.5 micron in-plane resolution, 250 micron z-axis slice thickness.

Administration of Magnevist In *Drosophila* Food Medium:

　　　500 μl Magnevist was prepared in 5ml total volume with water, and mixed with dry food medium until proper consistency. Approximately 3 mL was removed from an existing culture in the form of a slurry of larvae and food, which was then mixed into the new Magnevist CR medium.

　　　Flies reared in this medium showed no obvious effect on viability, and were found to live on the medium until the food supply was depleted.

Administration of Contrast Reagents by Direct Injection:

　　　Gadolinium contrast agent or manganese chloride solution was injected into larvae, pupae, and adults. Gadolinium contrast agent, but not manganese chloride solution, was injected into syncitial (early multinucleate stage) *Drosophila* embryos. Injection was accomplished using a drawn glass capillary needle, like those commonly used for injection of DNA into embryos. Results are as follows:

Embryos: Injection of 60-110 picoliters Magnevist solution (standard human preparation) into syncitial embryos produced no hatching larvae and appeared to have arrested development early. Manganese chloride was not tested.

Larvae and pupae: Green food coloring dye was diluted 1:12, and then mixed 1:1 with the CR for visualization of injected volume with a light microscope during the injection process. This solution was injected within the cuticle of 3rd instar larvae. In pupae, the above solution was injected both internally, into the abdomen of pupae, as well as into the interstitial air space which forms between the body of the developing fly and the pupal case itself which forms from the cuticle of the larva.

Results: When the interstitial space was filled, the fly did not survive and eclose from the pupal case, and development appeared arrested. A small volume injection, such as filling just the empty space anterior to the head, is less lethal, but also allows little access of the CR to the tissues of the developing fly.

When the mix was successfully administered internally, the fly did survive and appeared normal. Volumetric measurements of administered doses cannot be precisely measured with the current setup, and are between 100 pl & 1 μl; the volume was enough to easily visualize the green dye in concentrated drops which rapidly diluted as they were taken up by the fly's open circulatory system.

Adults: the same mix and quantity as above was injected into the abdomen. Flies survive and appear normal.

These results are summarized in the following table:

	Embryos	Larvae	Pupae	Adults
Gd Injection	Further Refinement Needed	Successful	Successful	Successful
Gd Food	Not Applicable	Successful	Not Applicable	Successful
MnCl₂ Injection	Not Tested	Further Refinement Needed	Successful	Successful
MnCl₂ in Food	Not Applicable	Further Refinement Needed	Not Applicable	Successful

Conclusion

Especially given the nature of the hardware used for these experiments, which was not designed for imaging, we were startlingly successful in our goal of producing high resolution virtually-dissectable 3D images of live *Drosophila* specimens, utilizing multiple imaging techniques. Based on these experiments we feel that the application of high-field MRI/MRS to *Drosophila* could represent an important direction for *in vivo* imaging and genetic modeling of human disease, that is worthy of further pursuit and technological development.

Additional Notes and Discussion

A possible lead for species and biomaterials studies is worth mention. *Drosophila* aracatacas generally yielded poorer imaging results in which the cuticle was less well defined than either *D. melanogaster* or *D. bifurca*. It may be noteworthy that *aracatacas* are an exceptionally sluggish, slow moving species, while *bifurca* is such an exceptionally quick species that is quite difficult even

to transfer flies from one vial to another without losing some of them. This suggests a question as to whether the material properties of the cuticle in these species that are necessary to support their physical dynamism produces an effect in imaging contrast, and that it may be interesting to study insect biomechanics with MRI.

Successful injection of embryos with contrast agent would be an exciting step forward. The possibility of injection of embryos with MRI contrast agents was not fully explored here, and offers fertile ground for future experiments. In general, the primary difficulty facing contrast agent experiments in model organisms is the challenge of how to actually get the contrast reagent to the interior of cells so they can be used as indicators of physiological states such as pH, calcium ion measurement and gene expression can be studied *in vivo*. *Drosophila*'s syncitial development has long been utilized for transgenic and RNAi methods, and could present a special opportunity for use of MRI contrast agents in a genetic model organism, due to the potential to load cells with contrast agent prior to cellularization. If an adequate concentration of contrast reagent is retained in the cells following subsequent divisions, the fly will have intracellular contrast agent in all its cells or only within specific cell lineages. In our preliminary attempt to inject embryos, no optimization was done to the formulation of the solution, and it was beyond the focus of our current effort to pursue the method further. Improvement of the buffer solution used for injection could lead to the development of an intracellular *in vivo* contrast agent system for the fly, a very exciting possibility. Such a method would provide an excellent opportunity for utilizing existing MRI contrast agents and encouraging the development of new varieties of contrast agents for molecular genetic studies.

Imaging with other nuclei:

Carbon imaging (C-13) on an untreated sample was attempted, but did not produce a successful image. Fluorine presents a stronger signal than carbon, and is not normally found in organisms naturally, so could be an interesting direction for imaging labeled molecules. Notably, bacteria can produce fluorinated proteins from fluorine-tagged amino acids, and thus might be used to synthesize *in vivo* labeling agents for use in fluorine imaging.

Economical high-throughput DNA extraction procedure in 96-well format for *Drosophila* tissue.

Holleley, Clare E. School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, NSW 2052, Australia. E-mail: c.holleley@unsw.edu.au.

One of the major benefits of working with *Drosophila* is the ease of achieving highly replicated and statistically powerful experiments. However in molecular studies, the time consuming process of single-tube DNA extraction can create a work-flow bottle-neck. We have modified the standard salting-out DNA extraction method so that it can be conducted in 96-well PCR plates, dramatically increasing the number of extractions that can be performed. The salting-out method is