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Supplement

Magnetic resonance virtual histology for embryos: 3D atlases for automated high-throughput phenotyping

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ABSTRACT

Ambitious international efforts are underway to produce gene-knockout mice for each of the 25,000 mouse genes, providing a new platform to study mammalian development and disease. Robust, large-scale methods for morphological assessment of prenatal mice will be essential to this work. Embryo phenotyping currently relies on histological techniques but these are not well suited to large volume screening. The qualitative nature of these approaches also limits the potential for detailed group analysis. Advances in non-invasive imaging techniques such as magnetic resonance imaging (MRI) may surmount these barriers. We present a high-throughput approach to generate detailed virtual histology of the whole embryo, combined with the novel use of a whole-embryo atlas for automated phenotypic assessment. Using individual 3D embryo MRI histology, we identified new pituitary phenotypes in *Hesx1* mutant mice. Subsequently, we used advanced computational techniques to produce a whole-body embryo atlas from 6 CD-1 embryos, creating an average image with greatly enhanced anatomical detail, particularly in CNS structures. This methodology enabled unsupervised assessment of morphological differences between CD-1 embryos and *Chd7* knockout mice ($n=5$ $Chd7^{+/+}$ and $n=8$ $Chd7^{+/-}$, C57BL/6 background). Using a new atlas generated from these three groups, quantitative organ volumes were automatically measured. We demonstrated a difference in mean brain volumes between $Chd7^{+/+}$ and $Chd7^{+/-}$ mice (42.0 vs. 39.1 mm^3, $p<0.05$). Differences in whole-body, olfactory and normalised pituitary gland volumes were also found between CD-1 and $Chd7^{+/+}$ mice (C57BL/6 background). Our work demonstrates the feasibility of combining high-throughput embryo MRI with automated analysis techniques to distinguish novel mouse phenotypes.

Introduction

In the wake of the first draft of the full mouse genome sequence (Mouse Genome Sequencing Consortium), large-scale mutagenesis programmes are underway (International Mouse Knockout Consortium) that will produce mice with gene knockouts for each of the approximately 25,000 genes in the mouse genome. Analysis of these mice in the coming years will give new insights into the genetic basis of human disease, as novel genes are identified that impact upon mammalian physiology and morphology. Mouse embryos in particular may be studied to determine the role of genes on development and congenital abnormalities. With an increasing number of new

Abbreviations: dpc, days post coitum; MRI, magnetic resonance imaging; OPT, optical projection tomography; μCT, microscopic computed tomography; DTI, diffusion tensor imaging; CNS, central nervous system; Gd-DTPA, gadolinium-diethylene-triamine-pentaacetic acid; VBM, voxel-based morphometry; SOD, septo-optic dysplasia; CHARGE, Coloboma of the eye, Heart defects, Atresia of the nasal choanae, Retardation of growth and/or development, Genital and/or urinary abnormalities, Ear abnormalities and deafness; SNR, signal-to-noise ratio; NSA, number of signal averages; TE, echo time; TR, repetition time.

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mutants, effective methods of identifying novel phenotypes in these embryos will be crucial.

Current phenotyping of embryo morphology is generally achieved by histological examination using microscopy. Specimens are dehydrated, wax embedded and thinly sectioned (2–8 μm) (Kaufman, 1992), providing high resolution 2D data and tissue sections that may also be stained for gene and protein expressions. Episcopic imaging is a development of this process, where autofluorescence of each tissue slice can be photographed and combined to generate high resolution 3D volume datasets (1–2 μm isotropic resolution) (Weninger and Mohun, 2002). However these histological approaches are time-consuming, introduce distortions into the final 3D image due to the sectioning process and do not readily enable rapid screening as only one embryo may be imaged at a time.

MRI is now an established method for non-invasive embryo imaging, beginning with the early work of Smith et al. (1994). High resolution 3D datasets with isotropic resolutions of down to 12 μm are created (Smith et al., 1996) with excellent soft tissue contrast, allowing the visualisation and segmentation of individual organ structures (Dhenain et al., 2001). MRI is also capable of high-throughput screening of multiple *ex-vivo* embryos (up to 32 in a single overnight scan) with the combination of a large volume imaging coil (Schneider et al., 2004) and fixation in an MR contrast agent (Cleary et al., 2009). Diffusion tensor imaging (DTI) — an alternative MRI technique — has also been used to investigate the structure of the embryo CNS by exploring the degree and direction that water is able to diffuse along neuronal axons (Zhang et al., 2003). Although this technique is ideal for investigating white matter, it is impractical for high-throughput imaging, as many hours are needed to generate connectivity maps in a single brain.

Other imaging methods such as optical projection tomography (OPT) and micro-computed tomography (μCT) are also able to non-destructively produce 3D datasets. OPT can create images of embryos that combine both anatomical structure and gene expression with conventional fluorophores, at high resolution (5–10 μm) (Johnson et al., 2006; Sharpe, 2004). However the technique requires embryos which are partially transparent thereby making its use challenging in older subjects (>13.5 days post coitum, dpc) (Schneider and Bhattacharya, 2004). μCT is also capable of acquiring high resolution datasets (typically less than 27 μm) in a short scan time (~2 h) (Johnson et al., 2006). Although conventionally μCT has difficulty in distinguishing soft tissues, which have inherently low contrast due to a narrow range of CT numbers (Holdsworth and Thornton, 2002), the use of CT contrast agents as tissue stains, such as osmium tetroxide (Johnson et al., 2006) and potassium triiodide (Degenhardt et al., 2010), have improved its ability to discriminate tissues. μCT is also particularly suited to skeletal studies, as it can produce excellent images of dense radiopaque structures such as bone (Oest et al., 2008).

Despite the availability of these advanced imaging techniques, any embryo dataset must still be manually assessed through inspection by a trained observer. As high-throughput analysis is increasingly demanded, conventional visual assessment for abnormalities is likely to become labour-intensive and insensitive.

Advanced computational techniques such as segmentation–propagation and voxel-based morphometry (VBM) have been used to investigate populations in both clinical and adult mouse MRI studies (Ashburner and Friston, 2000; Calmon and Roberts, 2000; Lerch et al., 2008; Sawiak et al., 2009). These methods enable anatomical differences between groups to be identified with little manual intervention or visual assessment. Integral to these techniques is the use of an *atlas*, a spatial average image of the whole population (Ashburner and Friston, 2000; Calmon and Roberts, 2000) created by finely warping individual subject images together to locally align anatomical features. Segmentation–propagation is a quantitative method for making volumetric measurements. After segmenting a volume of interest on the atlas image, such as the brain or heart, differences may then be identified between groups in the population by propagating the segmented volume to all individuals, thus providing the group mean and standard deviation from a single volume of interest on the atlas image. However, while average images of registered wild-type embryos have been reported previously (Zamyadi et al., 2008), there has so far been no application of atlas methods for phenotypic assessment. We envisage that a combination of multiple whole-embryo imaging with image processing techniques would allow the creation of an average embryo atlas from a population and enable automated phenotypic comparisons between transgenic and wild-type littermates.

In this study there were three stages to the investigation of our embryo atlas. We started by addressing the lack of brain tissue contrast on MR images. Initially we developed a contrast enhanced MR technique to produce structural detail in the embryo CNS. This was assessed in *Hesx1*$^{-/-}$ and *Hesx1*$^{I26T/I26T}$ mice, models of septo-optic dysplasia (SOD) (Dattani et al., 1998; Sajedi et al., 2008). Combining our contrast enhanced protocol with computational methods, we generated an MRI atlas for a population of CD-1 embryos and compared this against histology. Finally, this enabled the use of a segmentation–propagation technique to assess brain and cardiac phenotypic differences between CD-1, C57BL/6 strains and *Chd7*$^{+/-}$ knockout mice (a model of the condition CHARGE syndrome) (Bosman et al., 2005; Randall et al., 2009) based on a novel population atlas.

Materials and methods

Animal preparation

Pregnant female mice were sacrificed by cervical dislocation. Embryos were then dissected from the mother and transferred to warm Hanks solution. Their umbilical cords were cut and the embryos were allowed to bleed out into the solution. The embryos were then fixed in a solution of 4% formaldehyde and Magnevist MR contrast (Gadolinium-DTPA, Bayer-Schering Pharma, Newbury, UK) and left on a rotator. The embryos were removed and embedded in a 50 ml centrifuge tube using 1% agarose gel doped with an identical concentration of Magnevist to initial fixation. After comparing Gd-DTPA concentrations (2, 4, 8 and 16 mM) in groups of CD-1 embryos over two fixation durations (three days and two weeks), we determined that fixing embryos with 8 mM Gd-DTPA for at least two weeks produced the greatest SNR and brain contrast without major susceptibility artefacts. Three groups of mice were imaged at 15.5 dpc: outbred CD-1 mice from an established colony at UCL, and *Chd7* wild-type and heterozygous knockout mice (C57BL/6 background). Also *Hesx1*$^{-/-}$, *Hesx1*$^{I26T/I26T}$ and wild-type embryos at 18.5 dpc.

Imaging

Embryos were imaged on a Varian VNMRS 9.4 Tesla MRI system (Varian Inc., Palo Alto CA, USA) using a 33 mm quadrature birdcage volume coil (RAPID Biomedical GmbH, Würzburg, Germany). Maps of T_1 and T_2^* in tissue were created using a single sagittal slice field of view = 27×27 mm^2, 256^2 matrix size, 0.5 mm thickness. For 3D imaging we used a gradient-echo sequence, TR = 20 ms, 7 averages, and flip angle = 60°. Field of view = $27 \times 27 \times 27$ mm^3, matrix size = 512^3, zero-filled to 1024^3 on the console to a voxel-size of $26 \times 26 \times 26$ μm^3. Echo time (TE) was 9 ms for 15.5 dpc embryos and 5 ms for 18.5 dpc embryos to enable better visualisation of the bone structures of the cranial floor.

Image registration

Embryos were first semi-automatically extracted from the dataset and group-wise registration, using global and local transformations, was performed to create an average image atlas. The obtained deformation parameters from each individual to the template were then used to propagate the different regions of interest to each embryo.