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Quantitative In Vivo Imaging of Embryonic Development: Opportunities and Challenges

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Abstract

Animal models are critically important for mechanistic understanding of embryonic morphogenesis. For decades, visualizing these rapid and complex multidimensional events has relied on projection images and thin section reconstructions. While much insight has been gained, fixed tissue specimens offer limited information on dynamic processes that are essential for tissue assembly and organ patterning. Quantitative imaging is required to unlock the important basic science and clinically relevant secrets that remain hidden. Recent advances in live imaging technology have enabled quantitative longitudinal analysis of embryonic morphogenesis at multiple length and time scales. Four different imaging modalities are currently being used to monitor embryonic morphogenesis: optical, ultrasound, magnetic resonance imaging (MRI), and micro-computed tomography (micro-CT). Each has its advantages and limitations with respect to spatial resolution, depth of field, scanning speed, and tissue contrast. In addition, new processing tools have been developed to enhance live imaging capabilities. In this review, we analyze each type of imaging source and its use in quantitative study of embryonic morphogenesis in small animal models. We describe the physics behind their function, identify some examples in which the modality has revealed new quantitative insights, and then conclude with a discussion of new research directions with live imaging.

1. Introduction

Proper embryogenesis requires the successful completion of three fundamental processes: growth, differentiation, and organization (Dehaan and Ebert 1964). These three elements occur across length and time scales involving multiple cell types and secreted factors. Dissecting the functional contributions of each component and their molecular mechanisms is impossible without the assistance of small animal models for which visualization of these events can be achieved. Understanding has progressed slowly but deliberately over the last century, aided first through complex surgical manipulations and more recently well controlled genetic perturbations. Imaging technology has been essential to identify morphological changes in embryos over time and as a result of these perturbations. Early research studied large-scale changes in organ shape and/or growth that ultimately cause gross malformations that, in many cases, are not survivable in gestation or at birth. From this foundation, more focused and localized changes in tissue morphogenesis have been pursued, including conditional (time and space) genetic mutations, localized microsurgical ablation, and environmental changes.

Organs and tissues are organized through heterogeneous patterning by multiple cell lineages, each with the potential of multiple fate decisions. This has necessitated the development of reporter models and local labeling strategies (e.g. injectable dyes, micro-crystal particles).

Parallel advancements in experimental and imaging technologies are required to identify developmental phenomena and if possible quantify the resulting changes. Classical imaging of embryonic development is still performed through direct microscopic visualization or thin section preparations of fixed tissues. While acceptable for largely static tissues, fixation of dynamic tissues can result in atypical morphology that can be mistaken for malformations and some fixation processes can result in non-negligible and unpredictable changes in organ size. In addition, in-plane imaging of thin sections has greater resolution than along the transverse axis, creating difficulty in resolving highly tortuous anatomy. Even with time consuming three-dimensional (3D) reconstructions, only one data point in time for one embryo is obtained, necessitating multiple embryos with multiple experimental treatments to obtain statistical significance. As each surgery, injection, and/or genetic recombination event is not strictly identical, there is an increased risk of variability. This risk is elevated for rapid and/or dynamic processes that are sensitive to small changes in local cell phenotypes or environmental factors (growth factors, extracellular matrix, biochemicals, etc.) (Hogers, DeRuiter et al. 1999; Munoz-Sanjuan, Simandl et al. 1999; Maciaczyk, Singec et al. 2009). Furthermore, subtle malformations in early embryonic development can generate detrimental consequences in later embryogenesis and the ability to identify the origin of these malformations is critical. Continuous live imaging of embryonic morphogenesis would ensure that all data is obtained and available for analysis, but no imaging system to date can obtain the spatial resolution needed over the depth of field desired at a frame rate fast enough.

Currently there are four main imaging modalities for quantitative in vivo imaging: 1) optical 2) ultrasound, 3) micro-computed tomography (micro-CT), and 4) magnetic resonance imaging (MRI). Each of these imaging methods has differences in resolution capabilities, depth of field, acquisition time, and cost (Figure 1). In this review, we introduce each modality and discuss the physical rationale behind these trade-offs. We then offer guidance in balancing them for optimal performance in a specific imaging application. Finally, we conclude with new opportunities for imaging in embryonic development.

2.0 Optical Imaging

The compound and stereomicroscope are longstanding essential workhorses to assist in conducting experiments and qualitative observation. Compound microscopes focus light through transillumination while stereomicroscopes use incidence light to illuminate the sample. Visible light contains a wide range of wavelengths, each with different scattering properties affecting tissue penetration and quantification. Light is therefore filtered to single wavelengths (colors) and imaged digitally. This enables quantitative specific cell and molecular imaging through the use of excitable fluorophore tagged antibodies. Because filters remove all but the energy of the particular wavelength, the power of that light is often insufficient for anything other than thin sections. To address this drawback, lasers are used as the light source to produce much higher power at a single wavelength. Quantitatively visualizing multiple fluorophores (antibodies) in a sample is best achieved when each require a separate excitation wavelength (unique laser). It is possible to achieve excitation with a single laser if excited fluorophore emission can be filtered to specific wavelength bin (e.g. green, red, and far-red). With either microscope, the amount and usefulness of quantitative information is limited to tissue surfaces or very thin slices ($<10\mu\text{m}$) because of 1) the inability of visible light wavelengths to penetrate into dense media without scattering and 2) difficulties in partitioning light to different transverse-plane depths when creating two dimensional projections of the actual three dimensional tissue; therefore, while these approaches have achieved widespread use in live embryo imaging, they are primarily limited to virtually flat surfaces and/or qualitative observations of gross tissue movements.

2.1 Confocal Fluorescence Microscopy

Confocal fluorescence microscopy has been the most widely used optical imaging technique for quantifying live morphogenesis (Pawley 2006). Confocal microscopy uses point illumination delivered through a pin hole or line scanning. Line scanning utilizes the excitation of fluorophores in a sample to collect fluorescence through an objective, collecting data in a pixel by pixel manner throughout a plane of the specimen, taking approximately 1–10 μ s to acquire each sample (Khairy and Keller 2011). Pinhole scanning interrogates a three-dimensional slab of light simultaneously, increasing available light energy and amount of material in focus. Spinning disk confocal microscopy uses a rotating mirror to increase imaging speed, but reduces image quality for thick samples or lower powered objectives due to crosstalk between voxels (Khairy and Keller 2011). In general, confocal microscopy is fast and yields a high signal-to-noise ratio, but its depth of view is limited by the light scattering properties of the sample, which for embryonic specimens is a maximum of about 200 μ m.

Confocal microscopy has been used to track cell lineage (Bao, Murray et al. 2006; Murray, Bao et al. 2006) and gene expression patterns of *Caenorhabditis elegans* (Murray, Bao et al. 2006; Murray, Bao et al. 2008; Peng, Long et al. 2008; Liu, Long et al. 2009; Long, Peng et al. 2009). Additionally, gene expression maps have been created from the developing brain of *Platynereis dumerilii* (Tomer, Denes et al. 2010). Within vertebrates, confocal microscopy is often used to follow fluorescently labeled cell and matrix movements during early embryonic development. The embryos of fish (*Danio rerio* and *Oryzias latipes*) are particularly attractive for in vivo imaging because they are small, grow in nearly ambient environments, and are transparent through early cardiogenesis (Wittbrodt, Shima et al. 2002; Megason and Fraser 2003; Megason and Fraser 2007). Fish genetics are easily and quickly manipulated to create cell specific endogenous fluorescent labels. Farouhar et al utilized high speed confocal scanning to track early heart tube dynamics in 4D, and established that it functioned like a suction pump (Farouhar, Liebling et al. 2006). Similarly, Scherz et al used high speed confocal imaging to establish that zebrafish atrioventricular (AV) valve formation does not occur via a traditional endothelial to mesenchymal transformation, but through a folding of the endocardial layer at the AV junction (Scherz, Huysken et al. 2008).

Among higher order vertebrates, confocal microscopy enables highly detailed analysis of cell and matrix movements during primitive streak formation, gastrulation, and vasculogenesis. Avian embryos are particularly useful because their germ disk is virtually flat, very thin, and grows on top of the egg yolk permitting long-term optical access (Yalcin, A et al. 2010). Antibodies, injectable dyes, and transgenic labels have been used to track cell patterning and tissue morphogenesis. Charles Little, Brenda Rongish and colleagues have extended time-lapse confocal imaging to follow avian embryos from gastrulation up to heart tube formation and onset of beating (Czirok, Richard et al. 2005; Zamir, Czirok et al. 2006; Cui, Cheuvront et al. 2009; Sato, Poynter et al. 2010). They have quantified regional differences in cell and matrix fluxes during gastrulation and determined that autonomous (directed) cell movements occur, supporting that cells emerging from the primitive streak may already have gained initial specification (Zamir, Czirok et al. 2006). Brenda Rongish and colleagues have recently used confocal imaging in time lapse studies to follow autologous pre-endocardial cell movement, as compared to bulk convective tissue movement, in quail embryos during early heart tube formation finding that autologous cell movement makes a marginal contribution to heart tube development and fibrils of the extracellular matrix from the anterior lateral plate mesoderm move into the tubular heart (Figure 2) (Aleksandrova, Czirok et al. 2012). Through immunolabeling of the extracellular matrix components (fibronectin and fibrillin-2) and TIE1 positive endocardial progenitors, endocardial heart tube development was visualized through time lapse confocal microscopy. Separation and quantification of convective tissue velocity versus autonomous cell velocity

was established through the use of particle image velocimetry (PIV) techniques (Aleksandrova, Czirok et al. 2012). Mary Dickinson and colleagues implemented a novel slit scanning technique enabling fast imaging for dynamic analysis of early mouse heart development (Jones, Baron et al. 2004; Larina, Furushima et al. 2009). They determined that erythrocytes mobilize prior to the onset of cardiac contraction, but sustenance of the emerging vascular plexus requires hemodynamic force (Lucitti, Jones et al. 2007). Tissue scattering combined with limited ex vivo mouse embryo culture has prohibited confocal based imaging beyond the heart tube stage in mice (Jones, Crotty et al. 2002).

2.2 Multi-Photon Microscopy

Multi-photon microscopy (MPM) takes advantage of the nonlinear wavelength dependency of light scattering and absorption magnitude. For this type of excitation, two photons are absorbed instead of one, resulting in dramatic suppression of background noise. MPM can be used in most of the same applications as confocal microscopy, using infrared excitable fluorophores. Infrared light (< 800 nm) has dramatically reduced scattering through biological tissue, allowing deeper penetration (up to 2 mm) into live specimen samples with reduced toxicity (Denk, Strickler et al. 1990; Squirrell, Wokosin et al. 1999; Dickinson, Simbuerger et al. 2003; Diaspro, Chirico et al. 2005; Helmchen and Denk 2005; McMahon, Supatto et al. 2008; Supatto, McMahon et al. 2009). Like confocal microscopy, spatial resolution remains diffraction limited, but the $\sim 2\times$ longer wavelengths required limits resolution to approximately 400–500 nm. In addition, the lack of pinhole or line scanning options requires higher fluorophore density than confocal microscopy; thus, limiting imaging speed. Some of these limitations are overcome with the addition of light-sheet microscopy. Light-sheet microscopy projects micron thickness slabs of light orthogonally through a sample that illuminates structural details (Fuchs, Jaffe et al. 2002; Huisken, Swoger et al. 2004; Khairy and Keller 2011; Truong, Supatto et al. 2011). Harmonic MPM imaging allows for biological components to be visualized without the need of an exogenous label (Sun, Chu et al. 2004; Hsieh, Ko et al. 2008). Second harmonic imaging reveals ordered noncentrosymmetric structures such as collagen fibers, while third harmonic imaging recognizes interfaces of different levels of excitability such as cell membranes (Chu, Chen et al. 2003; Sun, Chu et al. 2004; Chen, Hsieh et al. 2006; Hsieh, Ko et al. 2008). In addition, a number of biological chemicals (e.g. NADPH) exhibit natural fluorescent emission that is captured by MPM with similar potency as exogenous contrast, enabling label free imaging and in some cases quantitative in vivo biochemistry (Zipfel, Williams et al. 2003).

Multi-photon microscopy has been used to study dynamic morphogenic processes and cellular patterning at deep tissue penetration and over long times within drosophila, zebrafish, avian, and murine embryos (Kulesa and Fraser 2002; Sun, Chu et al. 2004; Fowlkes, Luengo Hendriks et al. 2008; Hsieh, Ko et al. 2008). For example, Kyvelidou and colleagues used two photon imaging with third harmonic generation to track cell polarity and mitochondrial distribution in murine zygotes from the 2 to 8 cell stages (Kyvelidou, Tserevelakis et al. 2011). McMullen and colleagues used MPM to track the positions and identify the eventual fates of cardiac progenitor cells in the embryonic heart of mice in vivo following chamber segment formation (McMullen, Zhang et al. 2009). MPM has also been used to dynamically image atrioventricular and outflow tract pre-valvular cushions in embryonic chicks (Yalcin, Shekhar et al. 2010). Recently, Pantazis et al have developed harmonically excitable nanoparticles for multiphoton imaging with non-saturating intensity without bleaching or blinking (Pantazis, Maloney et al. 2010).

Optical imaging will continue to be the principle imaging tool for developmental biology inquiry, but with effectiveness limited primarily to early embryonic events. In vivo imaging

of later stage morphogenetic and tissue maturation events will necessitate the implementation of alternative imaging techniques with deeper tissue penetration.

2.3 Optical Coherence Tomography

Optical coherence tomography (OCT) is a non-invasive imaging modality that takes advantage of optical scattering in a specimen. It functions similar to ultrasound, but with light as the source instead of sound. Only the light that is reflected (thus coherent) from a sample at a certain depth is interrogated via an interferometer. A simple example of this in developmental biology is candling a bird egg to determine if it contains an embryo. Typically employing infrared wavelengths, OCT has a greater penetration depth into tissues than focused laser microscopy (up to 3mm) and is more flexible for producing dynamic 3D and 4D datasets across many different embryonic animal models (Figure 3) (Kim, Min et al. 2011) (Yang, Gordon et al. 2003; Liu, Wang et al. 2009; Choma, Suter et al. 2010). Though it has high spatial resolution ($\sim 10 \mu\text{m}$), OCT can suffer like ultrasound from multiple sources of reflectance in a specimen resulting in a low signal to noise ratio. Recent advancements in OCT technology have markedly improved signal strength, in particular using frequency domain imaging, emitting multiple optical frequencies simultaneously using an array (Spectral or Fourier-domain OCT), or sweeping the OCT source spatially (swept-source OCT). These improvements enable some of the fastest multi-dimensional scan rates possible. Over 100 2D frames/second and 5 3D volumes/second have been reported (Luo, Marks et al. 2006). OCT can be employed near limitlessly in embryos, as there is no requirement for fluorescence and all light is interrogated immediately after being emitted (Syed, Larin et al. 2011). Furthermore, OCT can be employed in a Doppler mode and scatter tracked via PIV algorithms to directly quantify cardiovascular hemodynamics (Jenkins, Peterson et al. 2010; S, D et al. 2011). Contrast media for OCT is currently limited to highly reflective materials like polystyrene microspheres and pyrolle nanoparticles, but significant efforts are underway to develop these further (Boppart, Oldenburg et al. 2005; Filas, Varner et al. 2011). One promising technique is spectroscopic-speckle variance OCT, where different molecular content can be assessed via a local shift in the speckle pattern independent of morphology. This was recently used in chick embryos to profile the transition between arterial and capillary blood supply in the developing chorioallantoic membrane (Liu, Zhang et al. 2011).

Originally implemented for retinal imaging (Fercher, Mengedoh et al. 1988), OCT has gained traction in developmental biology to study dynamic morphology in embryos (Yelbuz, Choma et al. 2002; Yelbuz, Wessel et al. 2004; Kagemann, Ishikawa et al. 2008; Manner, Thrane et al. 2009). This includes early epithelial fold deformations (Filas, Knutsen et al. 2008), early heart looping and contractions (Manner, Thrane et al. 2009), and later stage embryonic cardiovascular kinematics (Luo, Marks et al. 2006). Recently, Rugonyi and colleagues used spectral domain OCT to simultaneously quantify blood hemodynamics and tissue wall motions in the developing chick outflow tract, and found alterations in both profiles with conotruncal banding and vitelline vein ligation (Rugonyi, Shaut et al. 2008). Liu and colleagues rendered similar image datasets into finite element models to quantify local changes in fluid shear stress and wall mechanics during outflow tract development (Liu, Wang et al. 2009). Because commercially available OCT systems cost a fraction of the other systems profiled in this review, more use in embryonic imaging is likely as imaging speed, penetration depth, and contrast improve.

3.0 Ultrasound

3.1 Ultrasound Technology

Ultrasound imaging uses short pulses of high frequency sound waves which scatter when transmitted and/or reflected through tissue. This produces a characteristic “speckle” pattern with an intensity that is directly related to the local interference of the sound waves, or “echogenicity”. While usually associated with the plane being imaged, it is not uncommon for reflected out of plane sound waves to accumulate in plane, creating a “shadow” that partially obscures the view. The spatial resolution is half the pulse length; therefore, higher frequency transducers produce higher resolution images but lower frequency sound can penetrate deeper into tissues with less scatter (Lieu 2010). The lateral resolution is greatest at the narrow focal point of the sound beam. The ultrasound beam can be rapidly translated across planes through a mechanical sweep to generate 3D images, or more recently phased linear array transducers enable this to be done completely electronically with only slight reduction in in-plane resolution (Lieu 2010).

Ultrasound imaging has three performance modes: M-mode, B-mode, and Doppler. M-mode (motion-mode) uses a fixed transducer position and single beam direction transverse to a moving object such as the heart. It generates 1-dimensional spatial information (along the beam axis) rapidly with high temporal resolution of differential tissue lengths in the plane but M-mode cannot distinguish whether material is translating or rotating off the beam axis (Bushberg, Seibert et al. 2002). B-mode (brightness-mode) uses two dimensional echos returning from tissue boundaries to produce a digital signal proportional to the echo. This is displayed as a relative brightness based on the amplitude of the echo. A stronger echo from the tissue interface is seen as brighter sections in the image (Bushberg, Seibert et al. 2002). B-mode spatial resolution is dependent on the frequency of the beam and the frequency of the transducer sweep. Doppler ultrasound compares the incident and reflected frequency of a moving object (e.g. blood cells) to determine their velocity. The position and angle of the transducer with respect to the maximum velocity of the flow field is critical for accurate measurements, as the beam (and thus measurement) is 1-dimensional. Multidimensional (color flow) Doppler can resolve differences in blood velocity in space, but at a lower spatial resolution than B-mode (Bushberg, Seibert et al. 2002). Echocardiography can produce axial resolution of 0.05–0.1mm for B-mode and M-mode imaging and a lateral resolution for 0.2–0.5mm B-mode imaging (Zhou, Foster et al. 2002).

Using a transducer frequency greater than 30MHz is known as ultrasound biomicroscopy. Ultrasound biomicroscopy allows for the earliest stage of embryos (E 5.5) with transducer frequencies ranging from 30–40MHz to be imaged (Srinivasan, Baldwin et al. 1998; Phoon, Aristizabal et al. 2000; Foster, Zhang et al. 2002; Zhou, Foster et al. 2002; Zhou, Foster et al. 2003; Phoon, Ji et al. 2004). The cardiovascular system can be imaged using ultrasound biomicroscopy at an axial resolution of 30µm at E8.25 (Ji and Phoon 2005) but there is a reduction in the cardiac imaging planes from decreased penetration depth (Zhou, Foster et al. 2003). Exteriorizing the embryos combats this challenge (Phoon, Aristizabal et al. 2000; Phoon, Ji et al. 2004) but with a risk of affecting the hemodynamics and subsequent development.

3.2 Quantitative Ultrasound Based Phenotyping in Embryonic Development

Similar to optical imaging, ultrasound is inherently real-time. Deeper tissue penetration of the sound allows visualization of later embryonic and fetal stages. Furthermore, Doppler blood flow ultrasound imaging is a widely used experimental tool for analyzing the hemodynamic profiles in embryonic development (Figure 4). Ultrasound is useful to screen embryos for aberrant morphology and hemodynamic phenotypes, whether from targeted

genetic mutations or random mutagenesis experiments (Gui, Linask et al. 1996; Wessels and Sedmera 2003; Spurney, Leatherbury et al. 2004; Yu, Shen et al. 2004; Shen, Leatherbury et al. 2005; Yu, Leatherbury et al. 2008). Ultrasound imaging coupled with Doppler (3–7.5 MHz) is a standard method for assessing the fetal cardiovascular system clinically (Snider and Serwer 1990). Experimentally, studies date back over a decade using Doppler flow measurements with ultrasound biomicroscopy. Early development of ultrasound biomicroscopy systems demonstrated frequencies in the range of 40–50MHz for in vivo embryonic imaging (Turnbull, Starkoski et al. 1995). Other studies have demonstrated trans-abdominal measurement of cardiac development and function (Srinivasan, Baldwin et al. 1998). 40MHz transducers, coupled with continuous wave and pulsed Doppler, have been used for guided flow measurements in live mouse embryos (Christopher, Burns et al. 1996; Christopher, Burns et al. 1997). These technologies have since been combined for the development of an ultrasound biomicroscopy system which allows for continuous wave and pulsed Doppler hemodynamic measurements in normal and genetically altered live mouse embryos (Aristizabal, Christopher et al. 1998; Le Floch, Cherin et al. 2004; Ji and Phoon 2005). Due to the small size, rapid motions, and limited positioning control in mouse embryos within the mother, the hemodynamics of the dorsal aorta is largely used as a surrogate for intracardiac hemodynamics. Direct measurement of chamber specific blood flow and valve motions have been obtained largely from avian embryos (McQuinn, Bratoeva et al. 2007) (Butcher, McQuinn et al. 2007; Oosterbaan, Ursem et al. 2009). Ultrasound has also been used to quantify hemodynamic changes in experimentally manipulated avian embryonic hearts, most commonly via a metallic clip or ligation (Broekhuizen, Hogers et al. 1999; Ursem, Struijk et al. 2001; Ursem, de Vos et al. 2002; Stekelenburg-de Vos, Ursem et al. 2003; Stekelenburg-De Vos, Steendijk et al. 2005; Stekelenburg-de Vos, Steendijk et al. 2007; Hu, Christensen et al. 2009; Oosterbaan, Ursem et al. 2009).

3.3 Ultrasound Contrast

Ultrasound contrast agents are comprised of small gas bubbles encapsulated by a shell, typically ranging in sizes of a few microns (Qin, Caskey et al. 2009). The microbubbles are easily injected intravenously and remain in the blood flow, responding similarly to that of red blood cells (Jayaweera, Edwards et al. 1994). Ultrasound microbubbles are pulsed causing an increase and decrease in the diameter of the microbubble yielding a strong echo in perfused tissue (Dejong, Cornet et al. 1994; Dayton, Klibanov et al. 1999). Ultrasound manipulation of microbubbles coupled with optical imaging techniques have been used for in vivo two dimensional visualization of the microbubble oscillations (Klibanov, Ferrara et al. 1998; Dayton, Klibanov et al. 1999; Chomas, Dayton et al. 2000; Postema, van Wamel et al. 2005; Garbin, Cojoc et al. 2007). Ultrasound microbubble contrast agents have been studied for over the past 25 years and have been integral clinically but are not readily applied to embryonic model systems. If applied to embryonic systems, several different factors would have to be considered. Most importantly, the microbubbles would have to be in size range that could easily circulate in the vasculature without causing detrimental effects. Additionally, microbubbles are injected intravenously and remain within the circulation, which would limit the breadth of imaging that they could be used for in ultrasound. Microbubbles contrast agents have not been applied to developmental biology, it presents new research avenue to consider.

4.0 Micro-Computed Tomography (Micro-CT)

4.1 Micro-CT technology

Clinical computed tomography (CT) works by emitting an X-ray beam into a sample which scatters gamma particles in different directions. Clinical CT machines have up to 256

detectors that work simultaneously to capture a two dimensional image of the scatter pattern. This is ideal for a clinical setting because the system only required to rotate a small range. Extending from the principles of clinical CT, micro-computed tomography (micro-CT) has been used for the past 15 years to quantify complex spatial geometries at small resolutions (Butcher, Sedmera et al. 2007). The sample is scanned with high-powered X-ray energy from a single gamma emitter, whose attenuation is registered by a gamma camera opposite the emitter. Attenuation is quantified in Hounsfield units (HU) relative to a bone standard. The image is constructed through back projection based off of 360° discrete rotation angle increments which are a fully registered three dimensional near continuous series of planar slices with no registration defects using commercial software (Butcher, Sedmera et al. 2007). The contents of the entire cylindrical bore are imaged, but often a fast scout is performed to limit 3D reconstruction to a small region of interest. As X-ray wavelengths are extremely small, the spatial resolution from micro-CT is usually limited by the pixel density and acquisition dynamics of the gamma camera. Greater image density also requires rapid transmission and storage of large files. In vivo micro-CT has the ability to produce voxel sizes less than 10µm (Guldborg, Ballock et al. 2003) but X-ray exposure time is only a few minutes for a full-volume scan at approximately 25µm resolution (Kim, Min et al. 2011). Micro-CT systems have relatively inexpensive upfront costs and little overhead maintenance associated with them with a decrease in scan time as compared to other imaging modalities (Kim, Min et al. 2011).

4.2 Micro-CT imaging in embryonic development

While micro-CT has theoretically the highest possible imaging resolution, soft tissue attenuates X-rays very poorly; therefore, the vast majority of micro-CT imaging in embryonic development has been performed on fixed tissue specimens soaked with high atomic weight molecules that enhance X-ray attenuation (Metscher 2009; Degenhardt, Wright et al. 2010; Kim, Min et al. 2011). Osmium tetroxide provides the a high level of soft tissue contrast and clear tissue boundaries, but chemical diffusion is impaired by epithelial layers at later stages (Johnson, Hansen et al. 2006; Litzlbauer, Neuhaeuser et al. 2006; Bentley, Jorgensen et al. 2007; Zhu, Bentley et al. 2007; Faraj, Cuijpers et al. 2009; Metscher 2009; Kim, Min et al. 2011). Additional exogenous contrast used for embryonic soft tissues include Lugol's solution, iodine potassium iodide, galloxyanin-chromalum and phosphotungstic acid stains (Bentley, Jorgensen et al. 2007; Metscher 2009; Degenhardt, Wright et al. 2010; Kim, Min et al. 2011). With these methods, micro-CT has been used to create mathematical relationships for the volumetric growth of the heart, limb, eye, and brain in the chick embryo (Kim, Min et al. 2011). Alternatively, perfusion of a self-casting X-ray dense polymer (Microfil, Flow Tech, Inc.) into the luminal spaces of the embryo generates 3D lumen geometries for volumetric reconstruction and computational modeling (Butcher, Sedmera et al. 2007; Wang, Dur et al. 2009; Yalcin, Shekhar et al. 2011). None of these agents however, are suitable for live embryonic imaging.

Two different contrast agents, Omnipaque™(iohexol-OP) and Visipaque™ (iodixanol-VP), have been evaluated in recent studies as an in vivo CT contrast agent. OP has been used to visualize rabbit and rat placentas (Wise, Xue et al. 2010) and VP has been used to image in vivo human coronary arteries and to quantify kidney volumes in mice (Almajdub, Magnier et al. 2008; van Soest, Goderie et al. 2010). The toxicity of X-ray radiation on chick embryos and the contrast abilities and cytotoxicities of OP and VP have been measured (Henning, Jiang et al. 2011). Henning et al demonstrated that OP was embryotoxic 24 hours after injection regardless of the dose but 17 of 19 embryos injected with VP survived until the endpoint of the experiment at 10 days. This was likely because VP is iso-osmotic, while OP is hyper-osmotic. Organ volume measurements with live contrast enhanced embryonic images were identical to the control fixed tissue specimens treated with osmium tetroxide

(Figure 5) (Henning, Jiang et al. 2011). Additionally, VP produced contrast of 1,060 HU at 50 μ m resolution (bone is normally ~1000 HU). The radiation generated from micro-CT imaging has been a concern for inducing uncontrolled abnormalities in the embryos but Henning et al 2011 demonstrated that radiation up to 798mGray did not produce any defect for up to six days on chick embryos (Henning, Jiang et al. 2011).

With the resolution capabilities of micro-CT, studies have shown sub-cellular imaging in embryos but the development of non-lethal high magnitude contrast is required. Liposomal nanoparticle contrast agents for micro-CT (CT-LNP) can prolong the residence time and reduce cytotoxic effects in live animal imaging. Metallic nanoparticles, such as gold, have shown their efficacy as a viable contrast agent for micro-CT in previous studies (Murphy, Gole et al. 2008). Additionally, gold nanoparticles (50 – 200 μ m) can be encapsulated by a liposomal coating that increases the contrast, allowing for enhanced imaging of development. Varying parameters such as the size of the gold nanoparticle and the type of liposomal coating can have an effects on the toxicity, the tissue uptake, retention time, and level of contrast.

5.0 Magnetic Resonance Imaging

5.1 MRI Technology

Magnetic resonance imaging uses the inherent magnetic properties of atomic nuclei to generate an image. Anatomical imaging uses hydrogen ions for the signal source but functional imaging uses other ions such as carbon, oxygen, fluorine, sodium, and phosphorous (Driehuys, Nouis et al. 2008). First, the sample is placed in a large homogenous magnetic field (B_0) (Driehuys, Nouis et al. 2008). It is more energetically favorable for nuclei to align parallel to B_0 generating a net nuclear polarization. The polarization, density, and strength of the nuclear magnetic moments are the signal source for MRI (Driehuys, Nouis et al. 2008). A radio frequency (rf) pulse generates a short lived transverse magnetic field (B_1) that tilts the nuclear magnetization vector, through frequency resonance, into the transverse plane where it is detected (Bushberg, Seibert et al. 2002). The nuclear resonance frequency has a linear dependence on field strength; therefore, three linear, orthonormal magnetic field gradients encode spatial varying information building a three dimensional image (Driehuys, Nouis et al. 2008). Frequency and phase information for each image slice is collected and stored in the two dimensional matrix, k-space. An inverse Fourier transform of k-space for each slice gives a stack of two dimensional images which are interpolated into a three dimensional rendering (Bushberg, Seibert et al. 2002).

5.2 MR Contrast

MR contrast in soft tissues can be generated without the need of external contrasts through manipulation of spin relaxation events that occur after the rf pulse. After the rf pulse, the excited magnetization will re-align with B_0 according to the time constant T_1 (Bushberg, Seibert et al. 2002). The time between rf pulses is the repetition time (TR). Tissues having a T_1 greater than the TR do not recover as completely; therefore, will appear darker in the image (Driehuys, Nouis et al. 2008). This is known as T_1 weighting. The signal obtained from the rf pulse is a damped sinusoid where the exponential decay envelope function, described by the time constant T_2 , is tissue specific. The exponential decay is related to the loss of phase coherence from the micromagnetic inhomogeneities intrinsic to the individual spin-spin interactions of the nuclei. Each tissue type possesses their own intrinsic properties based on the physical and chemical nature of the tissue (Bushberg, Seibert et al. 2002). There is a time lapse, the echo time (TE), before the signal is recorded. Signals from different tissue types will have different intensities based on the amount of transverse magnetization that remains in them (Driehuys, Nouis et al. 2008). This is known as T_2

weighting. Alternatively, exogenous paramagnetic compounds such as gadolinium chelates can enhance MR contrast. Paramagnetic labeling of cells can be done using gadolinium chelate with a cationic liposome or microemulsion which has presently been done in mouse studies (Liu and Frank 2009). Super paramagnetic iron oxide (SPIO) nanoparticles have also been used to track single or clusters of labeled cells to target specific tissue populations (Liu and Frank 2009).

5.3 Quantitative Embryonic Imaging with MRI

MRI studies date back to the 1990's using fixed mouse embryos and close fitting hardware coils (Smith, Johnson et al. 1994; Smith, Linney et al. 1996). High field strength systems are used (7–11.7T), for volumetric analysis with a resolution of 20–50 μ m (Turnbull and Mori 2007). With gadolinium based contrast enhancement (Huang, Wessels et al. 1998; Aleksandrova, Czirok et al. 2012) and without (Dhenain, Ruffins et al. 2001; Schneider, Bamforth et al. 2003; Bamforth, Braganca et al. 2004) the cardiovascular system of fixed murine embryos has been imaged with MRI. Long scan times are required (6–24 hours) to generate the high resolution needed for embryonic imaging (Turnbull and Mori 2007); therefore, multiple embryo imaging (32 embryos at once) increases the throughput of phenotyping (Turnbull and Mori 2007). Common fixed studies include genotyping and phenotyping (Petiet, Kaufman et al. 2008; Zamyadi, Baghdadi et al. 2010) and contrast enhanced (Wadghiri, Blind et al. 2004). Furthermore, different acquisition protocol studies have been done which addresses the long scan time challenges (Schneider, Bamforth et al. 2003). Technical limitations hinder the ability of MRI to be used for live embryonic imaging. The resolution of the image is the limiting factor based on the signal-to-noise ratio (SNR) and scan time. Theoretically, the physical spatial resolution limit is 10 μ m but in practicality this limit is much larger to have adequate SNR with reasonable scan times. There are current rf coil configurations which are sufficient for murine and avian embryos, but these hardware systems work proficiently during mid to late stages of development. Advances in hardware would be needed to capture early stage development (Turnbull and Mori 2007). Additionally, undifferentiated and immature cell types in early development compromise the inherent contrast abilities of MRI (Turnbull and Mori 2007).

Due to non-periodic, bulk rotations of murine embryos, long scan times are required to reduce artifacts (Nieman, Szulc et al. 2009). The images can be registered together during post processing to eliminate some effects from motion (Maas, Frederick et al. 1997; Kim, Boes et al. 1999). Nieman et al corrected image artifacts using a six-parameter registration of the images. The rotations and translations are extracted from the data and approximate movements of the embryo are subtracted (Nieman, Szulc et al. 2009). Avni et al used the directionally dependent magnetization of blood for identification of different mouse embryos in utero. allowing for future phenotyping studies of the fetuses and placenta and possible vascular defects (Avni, Raz et al. 2011). This technique can allow for future phenotyping of the fetuses and placenta and identification of potential vascular defects.

Holmes et al determined cardiac and respiratory cycles in chick embryos using a self-gated cine MRI protocol and retrospective gating (Holmes, McCabe et al. 2008). Retrospectively, this protocol generates in ovo cardiac images that are devoid of aberrant data (Holmes, McCabe et al. 2008). This method could allow for multi-slice imaging, possibly serially images of the embryonic chick heart for different developmental stages (Holmes, McCabe et al. 2008). Hogers et al followed cardiovascular processes during early stage quail embryos (2.5–11 day) using a vertical 9.4T magnet and 30mm resonator coils (Figure 6) (Hogers, van der Weerd et al. 2009). The quail embryos was cooled to room temperature, reducing movement, and a venous clip at the right lateral vitelline vein was used to induce malformations (Hogers, van der Weerd et al. 2009). Rapid acquisition with relaxation enhancement (RARE) (Hennig, Nauerth et al. 1986) was used to acquire 300–500 μ m slices

with an in plane resolution of 78–90 μ m. Quality MRI images were obtained for development ranging from days 3–11 in the developing quail.

6.0 Image Processing

Each of the aforementioned in vivo imaging modalities is limited in spatial and/or temporal resolution, or otherwise does not achieve the depth of field. While many of these limitations are physical, a number of pre- and post-processing techniques have been developed to extend the power of each technique. These can be broken into three categories: 1) image acquisition 2) image restoration, and 3) image analysis (Khairy and Keller 2011).

6.1 Image Acquisition

For a dynamic system, specifying the timing of image acquisition – called gating – is essential to limit motion artifacts. Gating can be designed and performed during imaging (prospective), or established after the image dataset has been acquired (retrospective). Prospective gating synchronizes image acquisition to specific instances within a periodic (repetitive) signal emitted by the specimen. Because an electrocardiogram is not available in an embryo, usually the physical motion of the heart or large vessel is tracked. This has been achieved optically (Chan, Lin et al. 2009; Taylor, Saunter et al. 2011) or through simultaneous blood velocity tracking (Deng, Birkett et al. 2001; Herberg, Goldberg et al. 2005; Jenkins, Chughtai et al. 2007). Retrospective gating is achieved by acquiring all possible images in a dataset, and then sorting them based on periodic spatial or temporal signals. This approach has been employed for embryonic heart development imaging by taking advantage of unique non-symmetric myocardial cross-sections for each imaging plane and phase of cardiac cycle (Figure 7) (Liebling, Forouhar et al. 2005). Alternatively, a separate signal can be taken at the same time as the imaging, or extracted separately after imaging, and then used to separate each image. For example, Liu and colleagues derived spatially and temporally unique line scans from OCT image data to reconstruct 4D deformations in the avian outflow tract (Liu, Wang et al. 2009). Yoo and colleagues recently developed a spatial retrospective gating algorithm to increase the field of view and frame rate of an imaging series without compromising spatial or temporal resolution (Yoo, Larina et al. 2011). Using OCT, they translated their probe during imaging acquiring spatiotemporally overlapping fields of view, which could then be re-synchronized in time and space. They demonstrated a 6 fold increase in imaging frame rate in an embryonic rat heart. Retrospective imaging requires tremendous amounts of data to be rapidly acquired and stored, while prospective gating is data efficient but difficult to implement in an embryo.

6.2 Image Restoration

The objective of image restoration is to improve boundary detection and local contrast in an image dataset. Two key approaches are deconvolution and registration. Deconvolution of blurred objects is achieved through estimation of the spread of the data points and restoring them to their “true” locations (Lucy 1974; Markham and Conchello 1999). Point spread functions can either be assumed or derived directly from the datasets, the latter being the most computationally intense (Racine, Sachse et al. 2007). For image registration, spatially mis-aligned images are translated and/or transformed based on deviation from a defined reference frame (Khairy and Keller 2011). This reference can be an externally applied marker (e.g. hole or region of high scatter/contrast), or ascribed relatively through neighboring images (Brown 1992; Zitova and Flusser 2003). Both deconvolution and registration can and has been employed in the aforementioned gating processes to improve image quality.

6.3 Image analysis

The basis of image analysis is to translate the intensity of each image voxel into digital information that can be quantified and compared. For example, image segmentation delineates anatomic structures and tissue geometries, while thresholding bins the intensity of a voxel to assign values relevant to the study. Numerous algorithms have been developed and implemented for these purposes and are found in common imaging applications (e.g. NIH ImageJ), so the reader is referred to other sources for this information (Khairy and Keller 2011). Once obtained, these datasets can also be used in computational modeling, which simulates biological conditions to estimate local and global functional parameters that cannot be measured directly. For example, 3D and 4D geometry of embryonic hearts and outflow were used to quantify local changes in wall shear stress over development (Liu, Wang et al. 2009; Wang, Dur et al. 2009; Yalcin, Shekhar et al. 2011). More recently, efforts have expanded to connect finite element based mechanical deformation of embryonic tissues with regulating biological signaling (Brodland 2011), but this is still in its infancy.

7.0 Conclusions and Next Directions

Advances in in vivo imaging have transformed the study of embryonic development and contributed many new insights into dynamic processes of tissue and organ morphogenesis in a variety of animal model systems. In vivo imaging will continue to clarify which cells from what locations are responsible for positional cell fates and downstream signaling. Real-time and longitudinal analysis of cell migrations and differentiation will continue to help understand mechanisms of how initially two-dimensional sheets of uncommitted progenitor cells form three dimensional neo-organs with multiple related phenotypes and coordinated function. In parallel, identifying and tracking the evolving extracellular niche environments where local hubs of progenitor cell sequestration and differentiation occur will be essential to developing de novo tissue specific stem cell sources for regenerative medicine applications. In vivo imaging has the further capacity to quantify rates of cell migration, shape change, and differentiation in multiple dimensions, all of which would inform mechanical and biological mechanisms of tissue morphogenesis. Furthermore, direct quantification of solid and fluid motions in vivo will help understand how local fluid mechanics and tissue biomechanics contribute to and/or are affected by tissue morphogenesis.

The subcellular ($<1 \mu\text{m}$) resolution capacity of optical imaging is tempered by a depth penetration floor of about 3 mm. Conversely, whole animal multidimensional imaging technologies such as MRI and micro-CT lack cellular resolution ($<10 \mu\text{m}$). Though significant efforts have been made to close and bridge this gap with each technology, it is likely that data from multiple imaging modalities will need to be combined to understand the full picture of morphogenetic and functional mechanisms of organogenesis. Furthermore, the type of animal model being used directly correlates to the effectiveness of each imaging modality. Zebrafish are optically transparent and thin, making them an ideal model for optical imaging. Additionally, xenopus embryos can be imaged with optical techniques for early development but xenopus embryos also have pigmented epithelium which affects the optical penetration of the embryo. Avian embryos have a flat germ disc which allows for early development to be imaged with optical techniques but later stages of development are better captured with whole imaging modalities such as micro-CT or MRI. Murine models are large and dense which makes them non-ideal for optical techniques but easily imaged with micro-CT and MRI with appropriate contrast either exogenously or endogenously. Additionally, targeted molecular probes have dramatically expanded, strategies for local delivery of molecular and nanoparticle based contrast has not. Microinjection of liquid contrast currently clears quickly in embryos, but recent efforts suggest that nanoparticle size and shape could be tailored to diffuse more slowly and potentially accumulate in different

tissue regions over time (Gratton, Pohlhaus et al. 2007). This will be essential to visualize non-endothelial phenotypes and deep tissues in larger embryo models.

Finally, imaging technology is not just to view and quantify, but also can be used to intervene in embryonic development to non-invasively perturb cell and tissue morphogenesis. For instance, focused femtosecond laser photoablation has developed new understanding in the role of cell polarity and mechanotransduction in early cleavage and gastrulation events (Grill, Gonczy et al. 2001; Supatto, Debarre et al. 2005). In avian embryos, noninvasive photoablation through focused femtosecond laser pulses was achieved in the atrioventricular canal of embryonic chicks (Yalcin, Shekhar et al. 2010). Noninvasive photoablation can also be used to selectively activate conditional genetic mutagenesis, as was shown by Cambridge and colleagues in zebrafish and xenopus embryos (Cambridge, Geissler et al. 2009). Ultrasound is also able to noninvasively affect development. Endoh and colleagues implemented focused acoustic energy microbubble dispersion (the “shotgun” technique) to deliver DNA coding for luciferase or fluorescent protein expression locally in mouse embryos in utero without causing distal injuries (Endoh, Koibuchi et al. 2002). A related technology called ‘histotripsy’ uses focused ultrasound energy was to generate local tissue disruptions without affecting other tissues (Xu, Hall et al. 2007). Histotripsy was recently used to create a patent atrial septal defect in an adult canine (Xu, Owens et al. 2010), and local lesions within fetal sheep (Kim, Gelehrter et al. 2011). This technology is particularly attractive because of its potential for clinical translation, but current lesion size limits (400 μm) are not likely useful for very small or early stage embryos. Capitalization of these promising technologies for deeper understanding of morphogenesis and mechanisms of malformations will likely require partnerships between developmental biologists, physicists, and engineers.

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Figure 1.
Comparison of in vivo imaging modalities and their utility through early and late gestation in small animal models.

Figure 2.

Maximum intensity confocal image stacks in the dorso-ventral orientation immunostained with monoclonal antibodies for QH1 (red), B3D6 anti-fibronectin (green), and JB3 anti-fibrillin-2 (blue) of quail embryos at (A) HH8, (B) HH10, (C) HH11. (D) velocity vectors of Tie1::H2B-YFP pre-endocardial cells (green) and extracellular matrix proteins (fibronectin, fibrillin-2, red) based on displacement values for a 1 hour time period. The yellow square indicates 100μm (Aleksandrova, Czirok et al. 2012)

Figure 3. Structural and dynamic OCT imaging of early embryos (A) live 8.5 days post coitum (dpc) of an embryonic heart at 28 frames per second (Larina, Furushima et al. 2009) (B) 3D reconstruction of 9.5 days dpc embryo (Larina, Furushima et al. 2009) (C) diastolic sum voxel projection image (Jenkins, Chughtai et al. 2007) (D) 2D OCT coronal plane embryonic image during diastole (Jenkins, Chughtai et al. 2007)

Figure 4. Ultrasound images of HH17, HH21, and HH25 chick atrioventricular canals. Long-axis B-mode images, transverse axis M-mode images, and Doppler ultrasound of blood velocity through the AV canal is given. Scale Marks = 100 μ m (Butcher, McQuinn et al. 2007)

Figure 5. Contrast Enhanced Images with osmium tetroxide (OT), OP, and VP (A) 3D surface rendering and 2D cross sections (1–4) (B) 2D and 3D images injected with OT, OP, and VP (Henning, Jiang et al. 2011)

Figure 6. Sagittal slices of in vivo quail development using MRI. Development is given for days 3(a-b), 5(c), 7 (d), 9 (e), and 11(f). Scale bar = 5mm (Hogers, van der Weerd et al. 2009)

Figure 7. Image acquisition A) Non-gated 2D slice sequence acquisition before and after synchronization (Liebling, Forouhar et al. 2005) B) Mosaicing procedure for dynamic images (Yoo, Larina et al. 2011)