Prof. Rob deRuyter
Chairman of Biocomplexity Search Committee
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Dear Dr. deRuyter,

I would very much appreciate your considering my application for a professorship at the Biocomplexity Institute at Indiana University. Currently, I am a Senior Scientist in Biology and a Lecturer in Bioengineering at the California Institute of Technology. I am also a member of the Biological Imaging Center under the direction of Dr. Scott Fraser.

I have taken an interdisciplinary approach to studying brain and heart formation by combining the power of molecular genetics with the splendor of advanced optical imaging within living embryos. I have developed a transgenic avian model system and novel imaging methods that permit dynamic observation and manipulation of embryogenesis. My goal is to gain insight into the molecular and cellular mechanisms that drive normal forebrain morphogenesis so that I may better investigate developmental abnormalities such as holoprosencephaly.

I have included my curriculum vitae, research intentions, and have requested that letters of recommendation in support of my application be forwarded to the Search Committee.

Thank you for your time and considerations.

Sincerely,

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Proposed Research Projects

I. Molecular Imaging of Forebrain Formation

The vertebrate brain is likely the most complex structure ever assembled. Yet what is amazing is that throughout the animal kingdom, a myriad of new brains are created each day with exacting precision. My primary research interest is to study forebrain formation by combining the power of molecular genetics with the splendor of dynamic microscopic imaging. For several years I have been developing the tools and techniques to dynamically examine embryogenesis at the cellular and molecular levels in hopes of gathering enough information to begin to comprehend the enormous complexity of vertebrate brain formation.

It is my intention to dynamically visualize avian neurodevelopment at sub-cellular resolution by fluorescently labeling and optically recording all or nearly all the cells within a developing embryo. By recording cell and tissue movements during embryogenesis using multispectral, time-lapse laser microscopy and analyzing the data is using sophisticated software, I will be able to distinguish the subtle movements that thousands of individual cells make and identify the handful of genes that encode these events.

My goal is to gain insight into the molecular and cellular mechanisms that drive normal forebrain morphogenesis so that I may better investigate developmental abnormalities such as holoprosencephaly (HPE). HPE is a neurogenetic developmental disorder that may present as slightly as mild retardation to as dramatically as cyclopia (*Hum. Mol. Genet*. (1999) 8, 1683). A disruption of the sonic hedgehog-patched-GLI signaling pathway during early embryogenesis can result in HPE (*Nat. Genet*. (1996) 14, 357).

Sonic hedgehog (SHH) is a secreted signaling protein that performs a critical role in patterning of the head and brain. It is produced during embryogenesis by the cells of prechordal plate, and it results in the induction of ventral forebrain from the neural plate (*Cell* (1997) 90, 257). Following proteolytic cleavage, mature SHH binds to the PTCH receptor, and subsequent activation of the GLI transcription factors occurs. Experimental perturbation of the SHH signaling pathway in mice and chick embryos can be induced by gene inactivation (*Nature* (1996) 383, 407), by cyclopamine (*Development* (1998) 125, 3553), and by anti-SHH antibody (*Curr Biol* (1999) 9, 445) approaches. Grossly, it is known that cell types normally induced in the ventral neural tube by Shh are either absent or appear aberrantly at the ventral midline after cyclopamine treatment, while dorsal cell types normally repressed by Shh appear ventrally. The perturbed embryos will be visualized using dynamic multispectral imaging techniques in order to map cellular fates in this abnormal brain formation.

I will now briefly summarize two approaches that I intend to use to study forebrain formation as a faculty member at Biocomplexity Institute at Indiana University:

A. Production of transgenic fluorescent protein expressing quail to study forebrain formation

During the past five years, I have worked to develop transgenic, GFP-expressing Coturnix quail as an experimental system. Quail embryos are the ideal system to combine dynamic imaging and molecular genetics technologies. Quail reach sexual maturity quickly and are prolific breeders. The avian embryo is extremely hardy, permits unsurpassed accessibility for imaging and tissue manipulation, and has long been a favorite of classical embryologists.

Infecting primordial germ cells located within the blastoderm of unincubated quail eggs with retroviruses expressing GFP and a drug resistance marker can produce

transgenic quail. To increase the proportion of cells carrying the GFP gene in founder quail, drug selection is carried out within the eggs of injected avian embryos. I plan to generate transgenic quail that express various color variants of GFP from tissue specific and ubiquitously expressed DNA regulatory elements, thereby allowing their gene expression patterns to be dynamically recorded. The fluorescent protein expressing embryos will be indispensable for observing the tightly choreographed interactions between individual cells and populations of cells in normal and mutated embryos.

B. Refine approach for loss of function analyses in avians.

Targeted gene inactivation in mice is a common and powerful technique that researchers use to determine the functional significance of genes, but has yet to be developed for avian systems. RNA interference (RNAi) is widely used as a research tool to selectively erase the cellular contributions of individual genes to study their function. RNAi can occur when double stranded RNA that matches the mRNA produced by a given gene degrades that mRNA, thereby abolishing the function of that gene in a cell (*Nature* (1998) 391, 806). Short RNAi can be transcribed from an RNA polymerase III promoter by using RNAi expressing vectors or RNAi expressing retroviruses in mice (*Cell* (2002) **2,** 243; *Nat. Biotechnol.* (2002) **20,** 446; *Nat Genet*. (2003) 33, 401; (*Science* (2002) 296, 550) or in chick embryos (*Dev Growth Differ*. (2003) 45, 361; *Nat Biotechnol* (2003) 21, 93). RNAi's have been expressed endogenously in mice to silence gene expression resulting in phenotypes that recapitulate the gene knockout phenotypes (*Science* (2004) 303, 238; *Nat Biotechnol*. (2003) 21, 559).

By spatiotemporally limiting the RNAi expressing retroviral injections into the head region of stage 5-10 embryos, I will be able to compare RNAi mediated SHH inactivation in quail embryos with cyclopamine mediated SHH inactivation in quail embryos or SHH gene inactivation in mice. Inactivation of SHH in the developing head region of mice and avians leads to HPE. These embryos will be visualized using dynamic multispectral imaging techniques in order to map the cellular fates of abnormal brain formation.

C. Relevancy of my proposed research plans

My studies will permit progenitor cells that give rise to neuroblasts, angioblasts, and fibroblasts to be concurrently followed as they migrate and differentiate into neurons and glia, vascular, and cellular scaffolding, respectively, within the developing embryo. The proposed research will provide a more comprehensive understanding of the molecular and cellular basis of brain development, permitting better diagnosis of and rational therapeutic approaches for human 1st trimester brain development abnormalities. My proposed forebrain and holoprosencephaly research will significantly benefit from the interdisciplinary environment at the Biocomplexity Institute at Indiana University

Current Research Projects

II. Dynamic Visualization of Embryogenesis

My research aims to dynamically visualize and characterize avian embryonic development at sub-cellular resolution. My primary focus is determining how the brain and heart form and develop. Instead of labeling and following small numbers of cells at a time, I am have developed the means to optically record all or nearly all the cells within a developing embryo simultaneously. I typically try to place fluorescent tags within all avian embryonic cells using GFP-expressing retroviruses. Cell and tissue movements in the developing embryos are then recorded using multispectral, time-lapse fluorescent microscopy in 3D. Next the recorded data is analyzed using computers running sophisticated cell-tracking and color discrimination software capable of distinguishing the subtle movements that thousands of individual cells make and identifying a handful of genes that these cells express. The gene expression and cell migration data collected using laser microscopes is subsequently integrated within MRI collected datasets in order to understand the complex informational interactions that are occurring during development within the spatial and temporal context of the maturing embryo.

A. Production of transgenic GFP expressing quail

Developmental biologists have long used avians to study embryogenesis. Avians are often preferred to mice because the embryo is accessible at all stages of development. This accessibility makes them amenable to tissue transplantation and time-lapse videomicroscopy. However, the genetic techniques that have been so powerfully exploited in mice have not been developed for avians. So the crucial question is, is it easier to videorecord embryogenesis in mice or to introduce genetic techniques into avians?

It is my opinion that the best plan is to develop avian genetic techniques. Avian embryos are preferred because they are accessible to study at all stages of development without the heroic efforts that are required to study mouse embryos. Avians have long been the premier non-mammalian vertebrate model organism. The Japanese quail offers advantages in the small size of its egg, the moderate size of the breeding adults, and its short generation time. Because of these advantages, the proposed work permits molecular genetic experiments on a higher vertebrate embryo both more rapidly and less expensively than comparable work on the mouse.

Retroviral and expression vectors that co-express GFP and a selectable marker are being introduced into ES/PGC cells. Selection is arguably the most powerful force in biology. It governs species fitness and immune system B and T cell fitness. It can be used to isolate transfected cells that express resistance to certain drugs when grown in the presence of those drugs. Resistance to drug induced cell death imparts a selective advantage on the genetically modified cells. Ideally, the increased selective advantage will permit a higher degree of chimerism in the transfected embryos, which would lead to a higher frequency of germline transmission. Additionally, drug resistance can also be used to maintain gene expression is embryonic cells over time. By infecting blastoderm cells of stage X embryos (Hamburger and Hamilton 1951) with concentrated VSV-G pseudotyped GFP expressing retroviruses, I have been able to obtain >99% genetic chimerism in both quail and chick embryos. I am currently hatching putative transgenic quail to breed for germline transmission.

B. Tissue specific expression of GFP in avian embryos (with Charlie Little, Univ. of Kansas Medical School)

We have developed and are presently making several new GFP-expressing MoLV and HIV retroviruses. These retroviruses are self-inactivating which means that we delete much of the transcriptional machinery from the 3'LTR U3 region that is often recognized as foreign by the host cells and inactivated by methylation. We are also incorporating lox/Cre recombinase technology in order to remove most of the retroviral provirus from the infected cells' genome, leaving behind just the transgene. We are testing neural and cardiac specific transcription elements that display endogenous expression patterns when used as transgenes in mice. In addition, the transcriptional elements must be less than 2 kb is size in order to easily fit within the 9-10 kb size limitations of the retrovirus.

C. Dynamic Analysis of Blood Flow and Heart Development in Avian Embryos (with Mauri Gharib, Dept of Aeronautics at CIT and Kent Thornberg, Univ. of Oregon)

Our research is aimed at determining interacting roles of genetic programming and hemodynamic physical forces in regulating cardiovascular development in the early embryo. To capture blood flow in the developing avian we fluorescently label cells using various methods. Conventional laser microscopes are too slow to be able to collect XY images of blood flow or to resolve the cells within a beating heart without blurring. Digital particle image velocimetry (DPIV), a non-invasive, laser-based technique that enables accurate measurements of velocity in a plane, requires images without motion blurring. Test experiments show that frame rates of >300 frames/s are essential to reliable DPIV. We have designed and integrated a novel high speed imaging system in order to capture images of these fast-moving fluorescently labeled cells and tissues at frame rates of 300-500 frames/s.

We recently succeeded in recording and analyzing the first microscale cardiac electrical activity from an early embryonic (pre-looping) vertebrate heart. We monitored electrocardiograms from E2.5 quail embryos. A single recording electrode was positioned at various points adjacent to the heart (ventricle, A-V constriction, and atrium) with a second reference electrode in the egg albumen. Software tools we are now refining allow us to use the recorded ECG to assemble the individual images from the high speed imaging system into a full 4D image of flow patterns in the heart as well as the position/movements of the labeled cells that comprise the heart.

III. MRI of Quail Embryogenesis (with Melanie Martin, Seth Ruffins, and Russ Jacobs, CIT)

Numerous experiments have been carried out with an 11.7 Tesla magnetic resonance imager (MRI) to determine which parameters are the most useful to optimize image contrast in developing avian and mouse embryos. In MRI, signal intensity is obtained from water protons and is a function of the concentration/environment of water. MRI contrast results from variations in the environment that changes the characteristics of proton relaxation times. The T2-weigted 3D multi-spin echo routine used to collect images of murine embryos at different developmental stages (Dhenain et al., 2001) has also worked for quail embryos. Currently, the acquired 3D quail datasets have 20-50 um voxel resolution. We are able to take images of quail embryos in ovo and in vitro.

A. MRI Atlas of Quail Embryogenesis

Current atlases of embryonic avians are composed of micrographs of specimens processed by histological techniques, which is problematic due to problems of tissue warping and image alignment. We are using an MRI microscope for the acquisition of multi-modal volumetric data of live avian embryos in ovo. In brief, we are building and populating a software atlas for storing, viewing, searching, and sharing gene expression and cell migration patterns in the context of time-space collected images of quail embryos. This avian atlas can then be interactively rotated, computationally sliced, and then analyzed from any direction. This approach does not suffer from the warping and image alignment problems of all known histological approaches.

An important feature of the digital quail atlas is the ability to integrate other data types, such gene expression patterns and cell migration routes, into the anatomical models. There are several modes to input and integrate other data types. For example, we can superimposed or 'paint' gene expression derived from histological sections onto the models (Dhenian et al, 2001), we can assimilate cell migration data by overlaying images acquired using two-photon microscopy, or we can integrate gene expression patterns obtained in vivo using MRI by synthesizing an MRI contrast agent that is inactive until cleaved by beta-galactosidase (Louie et al., 2000).

B. Generating MRI contrast agents

(with Tom Meade (Northwestern Univ.) and Russ Jacobs, CIT)

MRI contrast reagents are able to alter the relaxation times of nearby water molecules and thereby enhance contrast. The alteration of relaxation times is due to direct interaction between the unpaired electrons of the paramagnetic ion and water protons. The development of bifunctional T2 contrast agents is the focus of the ongoing research. Specifically, contrast agents with both a superparamagnetic iron oxide core or magnetite and a fluorophore attachment are under development for applications in both light and MRI microscopy. In addition to the two imaging portions of these bifunctional agents, a directing molecule attached to the particle surface is also desirable. Such a directing molecule makes the contrast agent far more useful for imaging specific cell types, cell structures, and for many other applications. This MRI based approach permits structures to be imaged that are located too deep within the developing embryo to be seen by conventional light microscopy.

IV. BioNEMS (with Michael Roukes, Scott Fraser, and the DARPA BioNEMS team)

We are building a new family of biofunctionalized nanoelectromechanical systems (BioNEMS) that will carry the analysis of biological processes to the stochastic limit. To carry out these experiments, we are building a device with a large array of NEMS cantilevers (~500) in a small liquid volume (~100pL) capable of harvesting and analyzing the contents of a single cell within seconds.

My group has designed and synthesized alkanethiol molecules that are specifically attached in the form of self-assembled monolayers to gold pads located at the cantilever tips. We have developed the protocols to bind bacteria, virus, proteins, and nucleic acids to these molecules in a specific manner. We hope to be able to qualitatively and quantitatively compare the cellular components of individual cells within one another using this approach.

Past Research Projects

Multispectral imaging to resolve numerous fluorescent markers

(with Greg Bearman (JPL) and Scott Fraser)

We developed a new image detector that permits a plurality of fluorescent markers to be collected simultaneously instead of sequentially (Patent issued (US #6,403,332)). The approach (META) is now commercially available from Carl Zeiss, Inc.
2002 R&D 100 Award and the 2003 NASA Space Award
Publication: J Biomed Optics (2001) 6, 311-318.

Automated quantitative analysis of cell behaviors

(with Jerry Solomon and Steve Speicher, CIT)

We have created a computer cell tracking program, XVTrack, that can track and analyze individual cells or populations of cells in 3D from time-lapse data.
Publication: Current Biology 1997, 7:571–580.

Using retroviruses to fluorescently mark cells

We have generated over forty replication-defective, fluorescent protein expressing retroviruses to infect avian embryos. The fluorescent proteins have been tagged to direct their localization to specific cellular organelles, thereby allowing these structures to be specifically imaged, cell divisions to be followed, and morphological changes to be dynamically observed.
Publication: Exp. Neurol. (1999)156, 394-406; Nature Neuroscience (2003) 6(5), 507 – 518.

Blood island-derived stem cells contribute to intraembryonic vasculogenesis.

(with Mandy LaRue and Chris Drake, Medical University of South Carolina)

We showed that blood islands, the embryonic equivalent of the adult bone marrow, are a source of endothelial precursors that contribute to intraembryonic blood vessel formation (vasculogenesis).
Publication: Developmental Biology (2003) 262,162-72.

Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration

(with Catherine Krull, Scott Fraser, Marianne Bronner-Fraser (CIT) and Nicholas Gale, George Yancopoulos (Regeneron))

We demonstrated that Eph-related receptor tyrosine kinases and their ligands are essential for the segmental migration of trunk neural crest cells through the somites.
Publication: Current Biology 1997, 7:571–580.