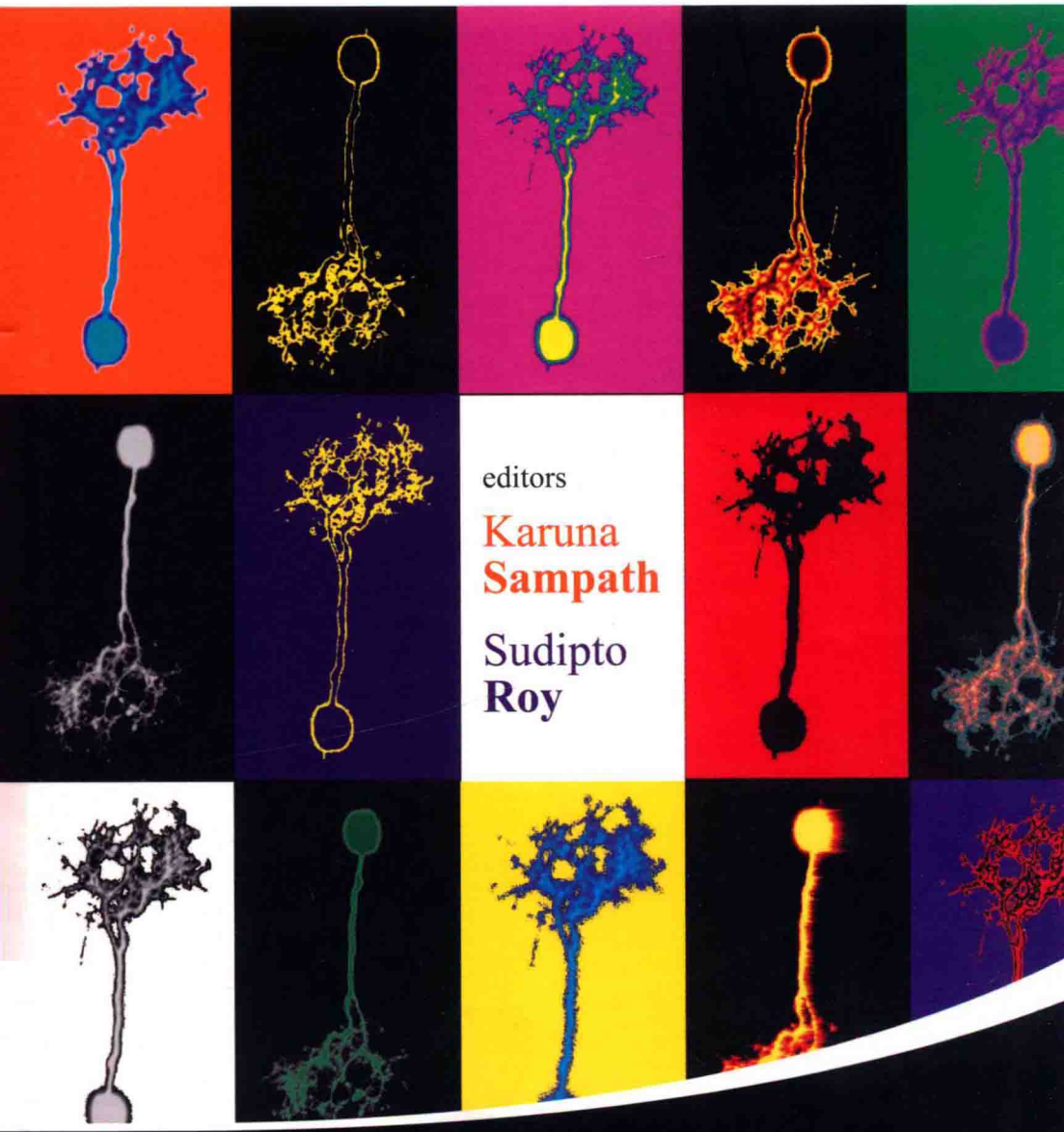
Live Imaging In ZEBRAFISH

Insights into Development and Disease



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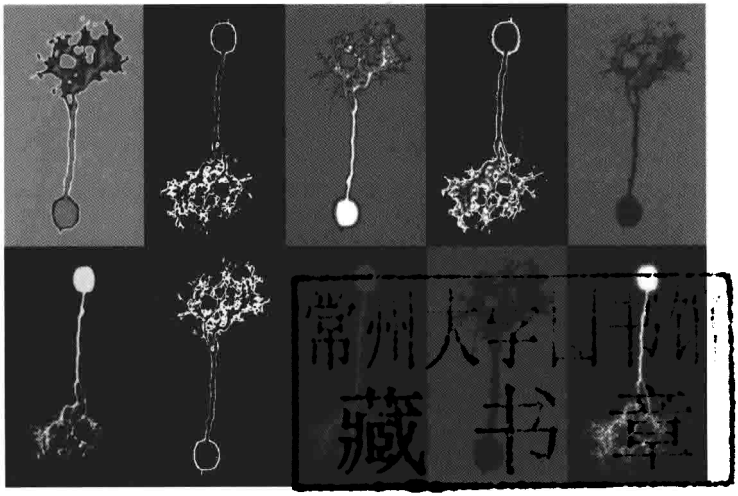

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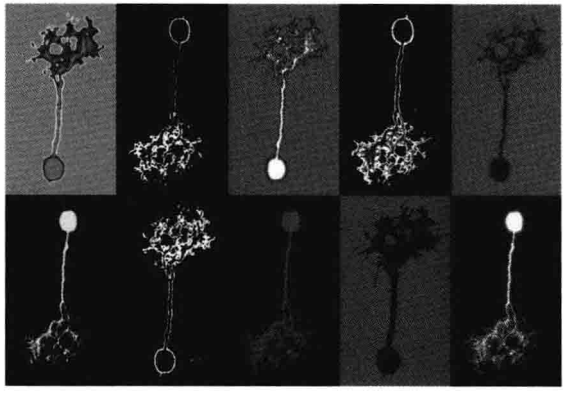

Preface

In the past two decades, the zebrafish, *Danio rerio*, has become an established and a widely accepted model for the study of embryonic development, and for understanding the cellular basis of various human diseases. One of the features of this model that has been widely publicized is the optical clarity of the embryos, and the potential for high-resolution microscopy of fixed as well as live samples. However, this salient feature has until recently, been rather underutilized. In order to highlight recent advances using real-time imaging in zebrafish, in this book, we bring together some outstanding examples of state-of-the-art imaging in the context of development, as well as infection and disease.

The first few chapters describe imaging of cell migration in the nervous system, both central as well as peripheral. The use of cell-type specific transgenes, new inducible expression systems, and novel enhancer/promoter cassettes are described in these chapters, with particular emphasis on expression in neuronal cells, oligodendrocytes, and glia. The chapter on fluorescence correlation spectroscopy describes this still very novel methodology, and one for which the zebrafish is particularly suitable. This technique allows real-time imaging and quantification at the single-molecule level, and has the potential to give biophysical insights into the formation of morphogen gradients and ligand-receptor interactions in living embryos. Another area that the zebrafish is now beginning to be appreciated for is its use in understanding organ physiology and function, and this is described in

the chapter on imaging digestive physiology. The last chapter covers imaging in the context of infection and wound healing.

We hope that readers working with the zebrafish in areas of developmental biology, cell biology and disease modeling will benefit from the methodologies and tools described in the book, and that it will be a valuable resource for students and researchers alike.

Finally, we thank all the contributors for their time and effort. We also owe special thanks to Ms. Joy Quek of World Scientific for her exceptional patience and hard work.

Karuna Sampath
Sudipto Roy
December 2009

Contents

Chapter 1

Analysis of Branchiomotor Neuron Migration in the Zebrafish

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Abstract

Cell migration plays an important role in a wide variety of biological processes. In the developing central nervous system (CNS), many neuronal precursor cells migrate from their site of origin to the final position in which they differentiate. In recent years, studies have often focused on stationary or *in vitro* systems to analyze neuronal migration. However, to understand the cellular and molecular mechanisms of neuronal migration, as well as the malfunction of this process in the diseased brain, it is of great importance to study it in real time in the live intact organism. Advances in microscopy techniques and the development of new dyes and genetically encoded markers have enormously improved *in vivo* time-lapse studies in the last few years. Here, we will describe the zebrafish as a model system to study the migration of a group of motor neurons in the hindbrain. These neurons move from the place where they are born and specified to more caudal regions in the brain. In order to study the cellular dynamics and molecular mechanisms of this process, we are using a stable transgenic line that expresses a green fluorescent protein (GFP) specifically in a subset of the hindbrain motor neurons. Using multiphoton excitation microscopy we can analyze their migration deep within the live tissue over extended periods of time.

Keywords: Zebrafish; Time-Lapse; Neuronal Migration; Central Nervous System; Two-Photon Microscopy.

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Introduction

Different Types of Neuronal Migration

Throughout development, the nervous system is undergoing major morphogenetic changes and the migration of cells plays an important role in these processes. The occurrence of neuronal cell migration was first noted in the developing cortex by Santiago Ramón y Cajal in the 1890s and was first experimentally addressed in the 1960s and 70s by Pasko Rakic, Mary E. Hatten and others. In the meantime, several groups of migrating neurons and several main types of cell migration in different regions of the developing and adult nervous systems have been identified. Neurons in the cerebral cortex, for example, undergo radial migration from their progenitor zone deep within the cortex to their final laminar position at the surface using radial glia cells, which span the entire cortex, as a substrate.[1] In addition to this radial type of migration, interneurons within different layers of the cerebral cortex have been shown to undergo tangential migration orthogonally to the direction of radial migrating neurons.[2] Finally, chain-like migration of neurons has been observed, e.g. for olfactory neuronal precursors, which are born in the subventricular zone (SVZ) and migrate to the olfactory bulb in close association with each other.[3] Interestingly, neurons adopt a different morphology depending on the type of migration they undergo. Radially migrating neurons typically exhibit a bipolar shape, with opposing leading and trailing process, and only transiently transform into multipolar shapes.[4,5] In contrast, tangentially migrating neurons dynamically change their morphology during their migration.[6] Despite these differences in neuronal morphology and migration, it has been suggested that the molecular and cellular mechanisms underlying neuronal migration are widely conserved.[7]

As of yet, the large majority of studies on neuronal migration have focused on *in vitro* dissociated cell culture systems or *ex vivo* observations in organotypic brain slices. However, to evaluate the relevance of these findings with respect to the *in vivo* situation, it will be critical to study neuronal migration by time-lapse video analysis in the intact organism.

Caudal Migration of Facial Branchiomotor Neuron (FBMN) in the Hindbrain

Cranial motor neurons control muscles involved in eye, head and neck movements, feeding, as well as speech and facial expression in humans. Mouse, chicken, and more recently zebrafish have been used as experimental model organisms to identify multiple genes that are involved in cranial motor neuron specification, differentiation, migration and axonal guidance.[8,9]

A subset of cranial motor neurons, the branchiomotor neurons (BMN), is specified in certain rhombomeres of the hindbrain. While most BMN will reside in the part of the hindbrain where they are born, neuronal precursors of the facial nerve (nVII), the facial branchiomotor neurons (FBMN), undertake a striking tangential migration along the rostrocaudal axis from rhombomere 4 (r4) to rhombomeres 6 and 7 (r6/7) (Fig. 1A). Despite their migration to r6/7, their axons will still exit the hindbrain at r4 to form components of the facial nerve nVII, which projects to branchial arch 4 and eventually innervates muscles of the head. Several genes have been identified that regulate the specification and migration of these neurons. These include the homeobox genes Hoxa1 and Hoxb1, which are key controllers of hindbrain segmentation and rhombomere identity. Mouse *Hoxa1*, and its zebrafish orthologue *hoxb1b*, are required for the segmental organization of r4, whereas murine *Hoxb1* and zebrafish *hoxb1a* regulate FBMN migration.[10,11] Furthermore, members of the *hedgehog* (*hh*) gene family have been shown to control the expression of the LIM-homeobox gene *Islet1* (*isl1*) and the homeodomain transcription factor Phox2b in postmitotic FBMN in vertebrates.[12] Finally, Tbx20, a member of the T-Box transcription factor family has been suggested to act downstream of *isl1* and *phoxb2*, to regulate a genetic program involved in FBMN migration in mice.[13]

In zebrafish, forward genetic screens have identified a number of mutants that affect caudal migration of FBMN. Interestingly, several of those genes have previously been associated with the non-canonical Wnt/Planar Cell Polarity (PCP) pathway,[12,14–17] which plays important

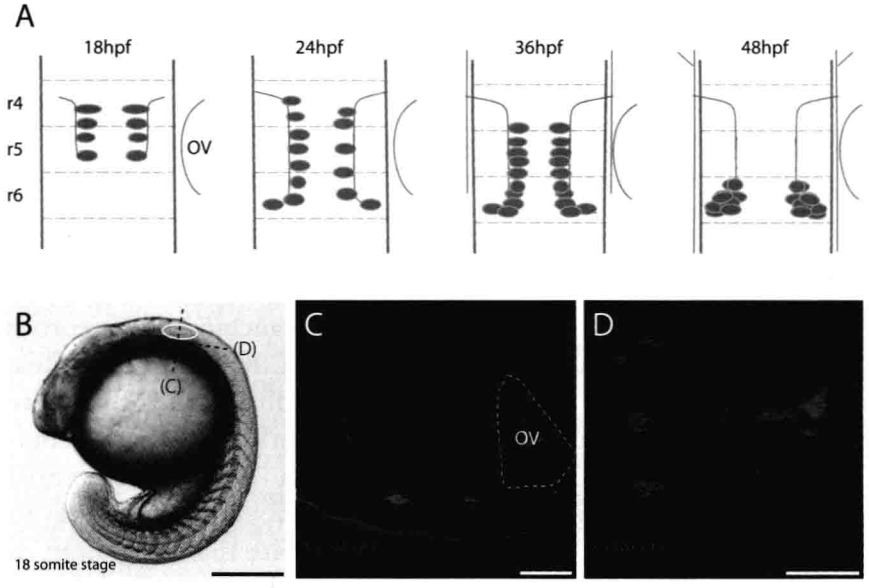

Fig. 1 Facial branchiomotor neurons (FBMN) in the zebrafish hindbrain undergo caudal migration during development. **(A)** Schematic drawing of FBMN migration in zebrafish. Dorsal views of the zebrafish hindbrain at the corresponding hours post-fertilization (hpf). The FBMN and their axons are shown in green and broken lines indicate rhombomeric boundaries (modified from Ref. 15). **(B)** DIC picture of a live 18 somite-stage embryo (18 hpf; lateral view). The otic vesicle (OV) is outlined in white and the plane of section for (C, D) is indicated with a dashed line. **(C)** A transverse section through the hindbrain at rhombomere 5 marked by the OV, shows the position of the FBMN close to the ventral midline (floorplate). **(D)** Coronal section through the hindbrain at the level of the FBMN migratory route. Anterior is at the top and posterior at the bottom. Filamentous actin (C, D) is labeled by phalloidin (red) and FBMN are labeled by the transgenic line *Tg(isl1:GFP)* (green). Scale bars: (B) 250 μm; (C, D) 30 μm.

roles in the planar organization of epithelial structures and morphogenetic movements in both vertebrates and invertebrates.[18–20] Genetic mosaic analyses have revealed that in the hindbrain, Wnt/PCP genes are predominantly required within the neuroepithelium surrounding the FBMN.[14–16] Thus, it has been speculated that Wnt/PCP signaling provides environmental cues involved in modulating the movement of FBMN from r4 to r6/7. Much less, however, is known about factors

that act within the FBMN to regulate their movement. An exception is the Wnt/PCP pathway component Prickle1b, which is expressed within the FBMN and cell-autonomously required for their migration.[21]

Importantly, until now the analysis of FMBN migration has focused on the identification of new genes and their function has predominantly been studied through stationary methods such as *in situ* hybridization or antibody staining. However, in order to understand the dynamics of FBMN migration and to identify the underlying molecular and cellular mechanisms, high-resolution time-lapse analysis of migrating motor neurons is required. In the following we will describe how two-photon (2P) live imaging can help in studying FBMN migration within the zebrafish hindbrain.

Imaging of FBMN Migration *In Vivo*

Zebrafish as a Model System to Study FBMN Migration

Why study FBMN migration in zebrafish? The zebrafish is a widely used vertebrate model system, which produces large numbers of embryos that undergo rapid development outside the mother. Because zebrafish embryos are transparent, cell behaviors and interactions can be easily monitored in live embryos using different microscopy techniques (Fig. 1B). Moreover, the availability of stable transgenic lines, such as the *Tg(isl1:GFP)* transgene to label FBMN,[22] allows the ready visualization of distinct populations of cells in the living organism. Finally, zebrafish are highly amenable for reverse and forward genetic tools, facilitating the functional analysis of genes involved in different developmental processes.

So far high-resolution live imaging of FBMN migration in the zebrafish has been sparse mainly due to experimental limitations. The FBMN are located relatively deep — around 100 μm — within the embryo on the ventral side of the neural tube, in close proximity to the floorplate (Fig. 1C). It is therefore difficult to image them with standard laser scanning confocal microscopy. One way to circumvent this problem has been to image hindbrain explants in culture,[23] however, it is not yet clear how much this culture system interferes with the

migration of FBMN. Thus, in order to image FBMN at high resolution within the intact organism, we have focused on adapting and developing microscopy techniques, such as 2P microscopy, suitable to monitor cells deep within the tissue over extended periods of time.

2P Microscopy as a Tool to Study FBMN Migration

The expression of fluorescent proteins is usually analyzed with standard laser scanning confocal microscopes to acquire high-resolution optical images. A key feature of confocal microscopy is its ability to produce in-focus images of thick specimens, a process known as optical sectioning. However, mainly due to the scattering of light through the tissue, the depth of observation is limited and it is thus difficult to study cells that are located deep in a tissue and/or have relatively low levels of fluorescence. Furthermore, confocal imaging can cause photobleaching and subsequent phototoxicity, limiting its use for time-lapse imaging of biological specimen. Multiphoton Fluorescence Microscopy is a relatively novel imaging technique in cell biology which provides two major advantages to regular confocal imaging.[24] First, it allows the generation of high-resolution three-dimensional images deep within live samples. This is because multiphoton microscopy uses higher frequency light, which can penetrate deeper into the tissue.[25] Secondly, photobleaching and phototoxicity are comparably low as multiphoton excitation only occurs at the focal plane[26] instead of in the entire light path through the sample as is the case in standard confocal microscopy.[27–29] There are also some limitations to using multiphoton microscopy. The image resolution obtained with 2P excitation is not better than that achieved in a well aligned confocal microscope and in thin specimens, photobleaching in the focal plane is higher as compared to conventional laser scanning microscopy.

Aspects of FBMN Migration Addressable by 2P Microscopy

Previous time-lapse analysis using standard laser scanning confocal microscopy has already provided some insight into the cellular basis of FBMN migration.[16,23] It has been shown that FBMN have

posteriorly-biased expansion and anteriorly-biased retraction zones, resulting in their effective posterior translocation. Moreover, analysis of FBMN migration in Wnt/PCP mutant embryos has provided evidence that mutant FBMN move more slowly and in random directions, and it has been speculated that this migration phenotype is caused by defects in the stability and/or polarization of their cell protrusions. However, to verify these observations, the spatial and temporal resolution of imaging FBMN migration and morphology still needs to be improved considerably. Questions regarding FBMN development that can be specifically addressed by 2P microscopy include: How do FBMN transform from a stationary epithelial neuronal precursor cell into a mobile one? How do they migrate through the epithelium? What is the substrate on which FBMN migrate? How do they interact with each other and/or the surrounding epithelium? What are the intrinsic and extrinsic factors determining FBMN migration? How and when is their initial polarity established, instructing them where to form dendrites and axons?

2P Microscopy Setup to Study FBMN Migration

In order to image the migration of FBMN in the live animal, a transgenic line expressing GFP under the control of a promoter/enhancer region of the transcription factor islet1 can be used.[22] This line specifically labels BMN after their final cell division and outlines BMN cell bodies and axonal processes including the peripheral branches within the muscles. By using this line, different aspects of FBMN development such as the initiation of migration, the type of movement, and the outgrowth and pathfinding of motor axons can be easily studied. To image the shape and behavior of the surrounding neuroepithelium during neuronal migration, the cell membrane of all cells in the embryo can be labeled by ubiquitously expressing a membrane-bound version of GFP (EGFPCAAX) or using a transgenic line (e.g. *Tg(β-actin:HRAS-EGFP)*), which drives ubiquitous expression of membrane-bound GFP.[30] Embryos are usually dechorionated at 18 hours post-fertilization, when the first FBMN are about to migrate caudally, and mounted within a self-assembled imaging chamber in low-melting point agarose to immobilize them (Fig. 2A). To further inhibit embryo motility, the

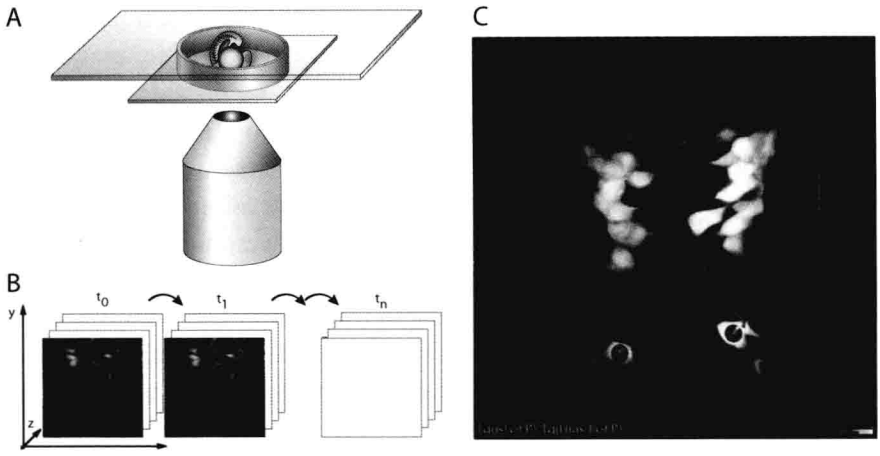

Fig. 2 Two-photon (2P) microscopy setup and image data analysis. **(A)** Mounting of embryos for imaging. Mounting chamber and embryo orientation used to image embryos on an inverted 2P microscope. The mounting chamber consists of a coverslip attached to a glass ring in which the embryo is placed in agarose medium. The region of the embryo that is going to be imaged (here the region of the hindbrain) should face the coverslip. **(B)** Image acquisition of a 4D data with *xyz* stacks obtained over *n* timepoints (*t*). **(C)** Image visualization, segmentation and analysis using Imaris software. The obtained 4D data sets are used for semi-automated tracking of cell shape and cell migration. The surfaces of the segmented objects are obtained and their centers are tracked over time. The final positions of the tracked FBMN are indicated by green dots and the movement of their center is visualized via color-coded tracks (color as a function of time).

anesthetic Tricaine is added to the agarose-containing mounting medium. For monitoring the movement and morphology of FBMN over time, we acquire a four-dimensional (4D) data set with high spatial and temporal resolution. Usually an XY resolution of 512 × 512 pixels with a scanning speed of 166 lines per seconds (lps) and a stack size of around 25 slices is sufficient (Fig. 2B). Since the speed of zebrafish development, and, as a result of this, of FBMN migration is temperature-dependent, we carefully control the imaging temperature through the use of objective heaters and imaging chambers.

For analysis of the 4D data set, we use several types of imaging software. Volocity (Improvision) and Imaris (Bitplane) are two