## DIVISION OF BEHAVIORAL NEUROBIOLOGY

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The vertebrate central nervous system (CNS) contains many different types of neurons that form at distinct characteristic positions and develop specific axonal connections and functions. This complexity has made it difficult to perform detailed functional analysis of neuronal circuits: in particular, it was very difficult to reproducibly identify cell types during investigation. For the past 15 years, however, molecular genetic studies have strongly suggested that the expression of transcription factors in the developing CNS helps determine the morphological and functional properties of neurons. This has opened up the possibility that researchers can use these transcription factors as markers to identify cell types in the CNS. Transgenic animals that express fluorescent protein in specific subsets of neurons are particularly powerful tools to study functions of the corresponding neurons in the neuronal circuits.

To fully exploit the methodology described above, we use larval zebrafish as experimental animals. The biggest advantage of this system is that larval zebrafish are almost completely transparent. This allows us to utilize many optical techniques, including morphological/functional imaging and optogenetics. We can also perform targeted in vivo electrophysiological recordings with relative ease in this transparent model. An additional advantage of zebrafish is that their CNS is much simpler than mammals. This enables us to perform detailed functional analysis of neuronal circuits at a single cell resolution. Our hope is to reveal operational principles of vertebrate CNS by using this simple system.

We have been focusing on studying neuronal circuits that control locomotion. Much of the control of locomotor movements is accomplished by neuronal circuitry located in the spinal cord. Therefore, the focus of our studies has been spinal neuronal circuits in larval zebrafish.

In addition to zebrafish, we have also started to use medaka as an experimental animal. Medaka has many advantages similar to zebrafish. Because NIBB is the main center of the Medaka National Bioresource Project, we are in a perfect place to conduct experiments using medaka. To begin with, we explored whether knock-in fish could be efficiently generated using the CRISPR/Cas9 system.

### I. Generation of Transgenic zebrafish

We have been generating transgenic zebrafish that express fluorescent proteins (GFP or RFP), Gal4, or Cre in specific classes of neurons in CNS by using promoter/enhancer of genes that are known to be expressed in subsets of neurons. Most of the genes we used are transcription factors expressed in subsets of neurons in the developing CNS. We also used those genes whose expressions are tightly related to neurotransmitter properties of neurons (i,e, vesicular glutamate transporter).

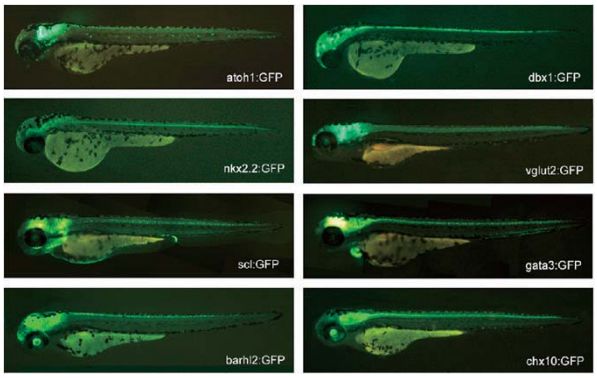


Figure 1. Examples of transgenic fish expressing GFP in specific classes of neurons.

In our early studies, we used a BAC-based transgenic technique for the generation of transgenic fish. In 2014, we succeeded in establishing a reliable knock-in method by utilizing the CRISPR-Cas9 system. The method we developed is highly efficient, such that nearly one-third of the raised animals become transgenic founders. Thus far, we have established more than 20 knock-in transgenic fish. The method greatly facilitates our functional analysis on neuronal circuits.

### II. Neuronal circuits that control rhythmic pectoral fin movements.

Limbed vertebrates exhibit coordinated rhythmic movements of forelimbs and hindlimbs during locomotion. Neuronal circuits that control rhythmic limb movements in mammals have been investigated for decades, but our knowledge is still limited because of the complexity of their limb. Rhythmic movements of pectoral fins during swimming in larval zebrafish is an attractive model (Figure 2). Pectoral fins of larval zebrafish show left and right alternated rhythmic movements, and they are actuated only by two types of muscles, i.e., abductor (Ab) and adductor (Ad) (Figures 3). Due to the simplicity of pectoral fins, we expect that we well be able to characterize neuronal circuits that control rhythmic pectoral fin more deeply.

We performed electrophysiological recordings of Ab motoneurons (MNs) and Ad MNs during fictive swimming. Both Ab MNs and Ad MNs show rhythmic spiking activitie (Figure 4). Activities of Ab MNs and Ad MNs on the same side essentially alternated. Voltage clamp recordings showed that both Ab MNs and Ad MNs received alternating excitatory and inhibitory inputs in a swimming cycle. Excitations mainly occurred in their preferential firing phase, and inhi-

bition mainly occurred for the rest of the period. To obtain insights into the source of these inputs, we are now investigating timings of spiking activities in possible premotor interneurons.

Figure 2. Rhythmic movements of the pectoral fin (arrow) during swimming in larval zebrafish.

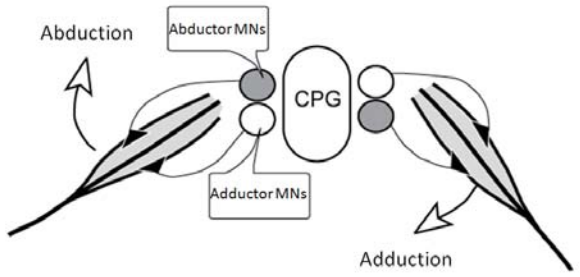


Figure 3. Schematic of rhythmic movements of pectoral fins during swimming. CPG, Central Pattern Generator.

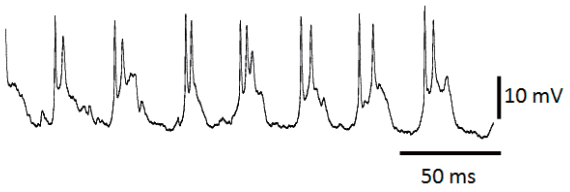


Figure 4. Rhythmic firings of an abductor motoneuron during swimming.

## III. Functional analysis of En1-positive neurons for axial movements during swimming.

Inhibitions play important roles for shaping motor outputs during locomotion. In the spinal cord of larval zebrafish, there are mainly two types of inhibitory neuron: commissural inhibitory neurons and ipsilaterally-projecting inhibitory neurons. The role of the former (commissural inhibitory neurons) is easy to understand: they are likely to play important roles for ensuring antagonistic movements of the left and the right side of body. The role of the latter (ipsilaterally-projecting inhibitory neurons) is less clear.

En1-positive neurons constitute major components of ipsilaterally-projecting inhibitory neurons in the spinal cord. To investigate the function of En1-positive neurons, we have genetically-ablated En1-positive neurons by using the Cre-loxP system (Figure 5A). In the resultant larvae, the cycle period for the rhythmic bending of the body was prolonged (Figure 5B), indicating that swimming speed was slowed down. The results show that En1-positive neurons play an important role for controlling locomotion speed.

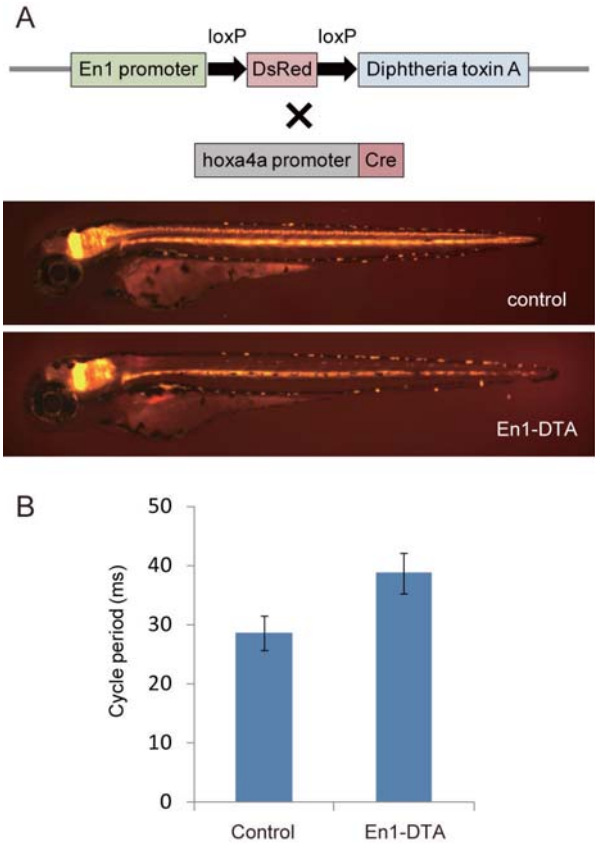


Figure 5. Spinal En1 neurons play an important role for controlling swimming speed. A, Ablation of spinal En1 neurons by using the Cre-loxP system. DTA, diphtheria-toxin A. B, Cycle period of the motor bursts during regular swimming in control and En1-DTA larvae.

## IV. Axially-confined in vivo single-cell labeling by primed conversion using blue and red lasers with conventional confocal microscopes

Green-to-red photoconvertible fluorescent proteins (PCFPs), such as Kaede or Dendra2 are powerful tools to highlight a small number of cells in a globally fluorescent cellular context. However, perfect single-cell labeling in tightly packed tissues such as those that make up the nervous system has been difficult because the traditional method for efficient photoconversion requires the use of violet/UV single-photon excitation (i.e., 405 nm), which is not confined to the axial dimension. Under conditions where many cells express PCFPs in a three-dimensional manner, cells that are located above and below the target cells undergo photoconversion when exposed to a single-photon converting beam.

Recently, Dendra2 has been found to undergo efficient photoconversion by a new method termed primed conversion that employs dual wave-length illumination with blue and red/near-infrared light. By modifying a confocal laser-scanning microscope (CLSM) such that two laser beams only meet at the focal plane, confined photoconversion at the axial dimension has been achieved. The necessity of this custom modification to the CLSM, however, has precluded the widespread use of this method.

We investigated whether spatially-restricted primed

conversion could be achieved with CLSM without any hardware modifications. We found that the primed conversion of Dendra2 using a conventional CLSM with two visible lasers (473 nm and 635 nm) and a high NA objective lens (NA, 1.30) resulted in a dramatic restriction of photoconversion volume: half-width half-maximum for the axial dimension was below 5 μm, which is comparable to the outcome of the custom method that employed the microscope modification. As a proof of this method's effectiveness, we utilized this technique in living zebrafish embryos and succeeded in revealing the complex anatomy of individual neurons packed between neighboring cells. Because unmodified CLSMs are widely available, this method can be widely applicable for labeling cells with single-cell resolution.

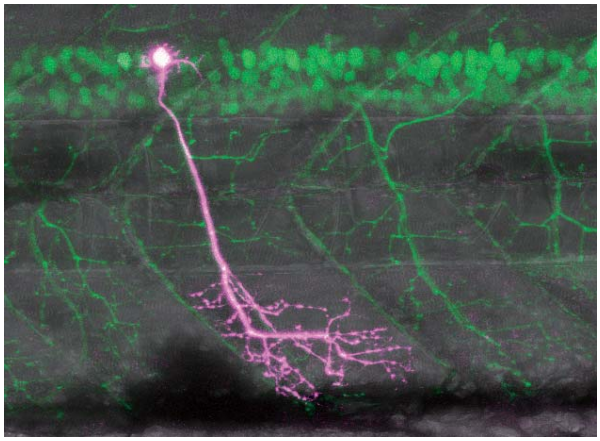

Figure 6. Primed conversion of Dendra2 reveals the fine morphology of a single motoneuron in a larval zebrafish

## V. Highly efficient generation of knock-in transgenic medaka by CRISPR/Cas9-mediated genome engineering

As mentioned above, we have established an efficient (~30%) knock-in system via non-homologous end joining (NHEJ) using the CRISPR/Cas9 system in zebrafish. If the same technique were applicable in medaka, it would greatly expand the usefulness of this model organism. This point, however, has not yet been addressed.

We report the highly efficient generation of knock-in transgenic medaka via non-homologous end joining (NHEJ). Donor plasmid containing a heat-shock promoter and a reporter gene was co-injected with a short guide RNA (sgRNA) targeted for genome digestion, an sgRNA targeted for donor plasmid digestion, and Cas9 mRNA. In approximately 25% of injected embryos, broad transgene expression in the expression domain of a target gene was observed. By raising these animals, we succeeded in establishing stable knock-in transgenic fish with several different constructs for five genetic loci. The efficiencies of obtaining transgenic founders among the raised animals exceeded 50% for all five loci. Further, we show that the method is useful for obtaining mutant alleles. In the experiments where transgene integrations were targeted between the transcription start site and the initiation methionine, the resultant transgenic fish became mutant alleles.

With its simplicity, design flexibility, and high efficiency, we propose that CRISPR/Cas9-mediated knock-in via NHEJ will become a standard method for the generation of transgenic and mutant medaka. (Watakabe, I. et al. Zool. Lett. in press)

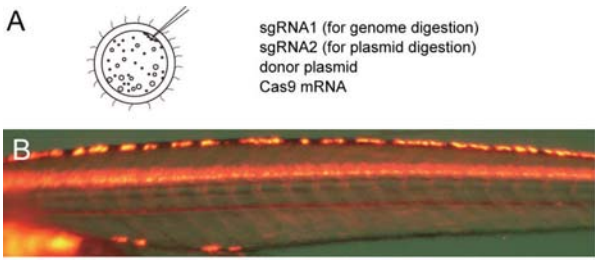


Figure 7. (A) For the generation of knock-in transgenic fish, sgRNA1 (for genome digestion), sgRNA2 (for plasmid digestion), donor plasmid with a bait sequence, and Cas9 mRNA are co-injected into one-cell-stage medaka embryos. (B) An example of transgenic medaka in which RFP was knocked-in into the vacht gene. As with the endogenous gene, RFP is expressed in motoneurons.

### Publication List:

〔Original Papers〕


- Ratanayotha, A., Kawai, T., Higashijima, S., and Okamura, Y. (2017). Molecular and functional characterization of the voltage-gated proton channel in zebrafish neutrophils. Physiol. Rep. *15*, e13345.
- Taniguchi, A., Kimura, Y., Mori, I., Nonaka, S., and Higashijima, S. (2017). Axially-confined in vivo single-cell labeling by primed conversion using blue and red lasers with conventional confocal microscopes. Develop. Growth Differ. *59*, 741-748.