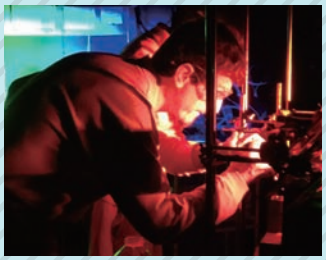
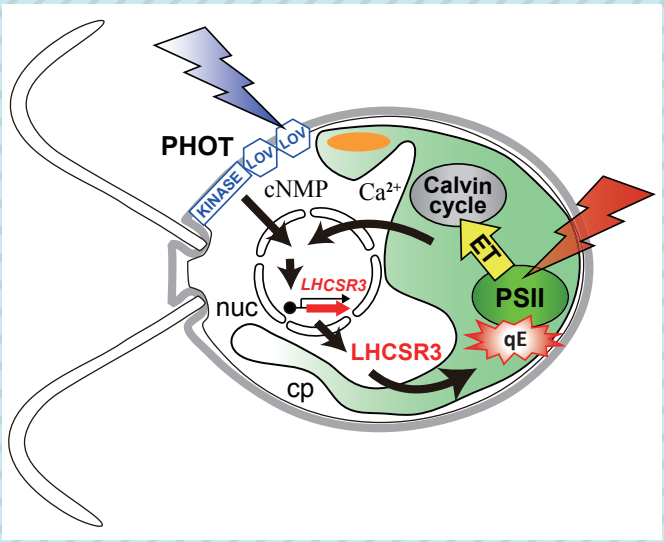
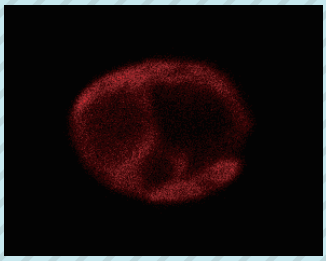
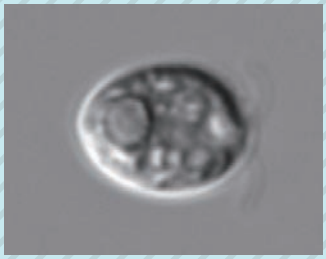








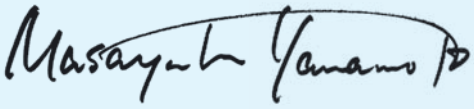
National Institute for Basic Biology

2016 ANNUAL REPORT

CONTENTS

The cover items are related to a paper titled "A blue light photoreceptor mediates the feedback regulation of photosynthesis" (Petroutsos, D., Tokutsu, R., *et al*., Nature 2016) from the laboratory of Prof. Minagawa. The paper demonstrated the existence of a molecular link between photoreception, photosynthesis, and photoprotection in the green alga *Chlamydomonas reinhardtii*. See page 66 of this report for details. See also page 79 for the instrument "Okazaki Large Spectrograph" used in this research.

INTRODUCTION



It is my great pleasure to introduce to you all the 2016 Annual Report of the National Institute for Basic Biology (NIBB), which outlines the research activities of the Institute and its effective function as a center for collaborative research in Japan over the last year. Given these important missions of the Institute, I recognize my strong responsibility to maintain the liberal and forward-looking atmosphere for research and discussion that NIBB has kept for many years, which I believe is a basis for these remarkable activities.

2016 was a marvelous year for us all, as Dr. Yoshinori Ohsumi, a Professor Emeritus of NIBB, won the Nobel Prize in Physiology or Medicine for his outstanding research on autophagy. He ran his laboratory as a Professor in NIBB from 1996 to 2009, and he recalls this period as the golden age of autophagy study. We congratulate his great achievements, and are proud of the efficacy of NIBB that fostered his science.

The Japanese government continues to ask universities and inter-university research institute corporations, the latter of which includes NIBB, to strengthen performance and gain profits from science. It is also necessary for the scientific community to achieve full compliance and establish integrity in research activities, to recover society's reliance on science. Under these circumstances NIBB must stride properly. We will maintain two directions steadily. One is that every person in NIBB should do his/her best in accomplishing good research in basic biology. Good science, even in a basic research field, will eventually benefit human beings. The history of science tells us this is true. The other is that NIBB must achieve high ethical standards for research that conform to the era of computer technology and data sharing through the internet. NIBB will work hard to be truly acknowledged as a remarkable institution by society.

Please find in this booklet a summary of the research, collaborative, educational, and international activities of NIBB in 2016. I would like to note that we welcomed several new colleagues in 2016, including four professors, one specially appointed professor, one associate professor, five assistant professors, one specially appointed assistant professor, and one NIBB research fellow, while nine colleagues transferred to other institutes.

Besides Prof. Ohsumi, I would like to congratulate Professor Mitsuyasu Hasebe for winning the Japan's Eminent Evolutionalist Award, and Dr. Yoshiaki Nakamura for winning an Outstanding Presentation Award from an academic society, as detailed on page 7.

To establish NIBB as an international leading institute in the field of basic biology, we always welcome your suggestions, comments and queries concerning our activities, in addition to your warm support.

Masayuki Yamamoto
Director General of NIBB
November 28, 2017

ORGANIZATION OF THE NATIONAL INSTITUTE FOR BASIC BIOLOGY

The National Institute for Basic Biology (NIBB) was founded in 1977 on a hill overlooking the old town of Okazaki in Aichi prefecture as one of the Inter-University Research Institutes to promote and stimulate studies of biology. NIBB conducts first-rate research on site as well as in cooperation with national, public and private universities and research organizations. NIBB attempts to elucidate the general and fundamental mechanisms underlying various biological phenomena throughout a wide variety of biological fields, such as cell biology, developmental biology, neurobiology, evolutionary biology, environmental biology, and theoretical biology.

NIBB and four other national institutes, the National Astronomical Observatory of Japan (NAOJ), the National Institute for Fusion Science (NIFS), the National Institute for Physiological Sciences (NIPS), and the Institute for Molecular Science (IMS), constitute the National Institutes of Natural Sciences (NINS), which is one of the four Inter-University Research Institute Corporations. NIBB's activities are supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. The Okazaki Institute for Integrative Bioscience was established as a common facility for the three institutes (NIBB, NIPS, and IMS) of the Okazaki campus.

Buildings in the Myodaiji area (left) and Yamate area (right).

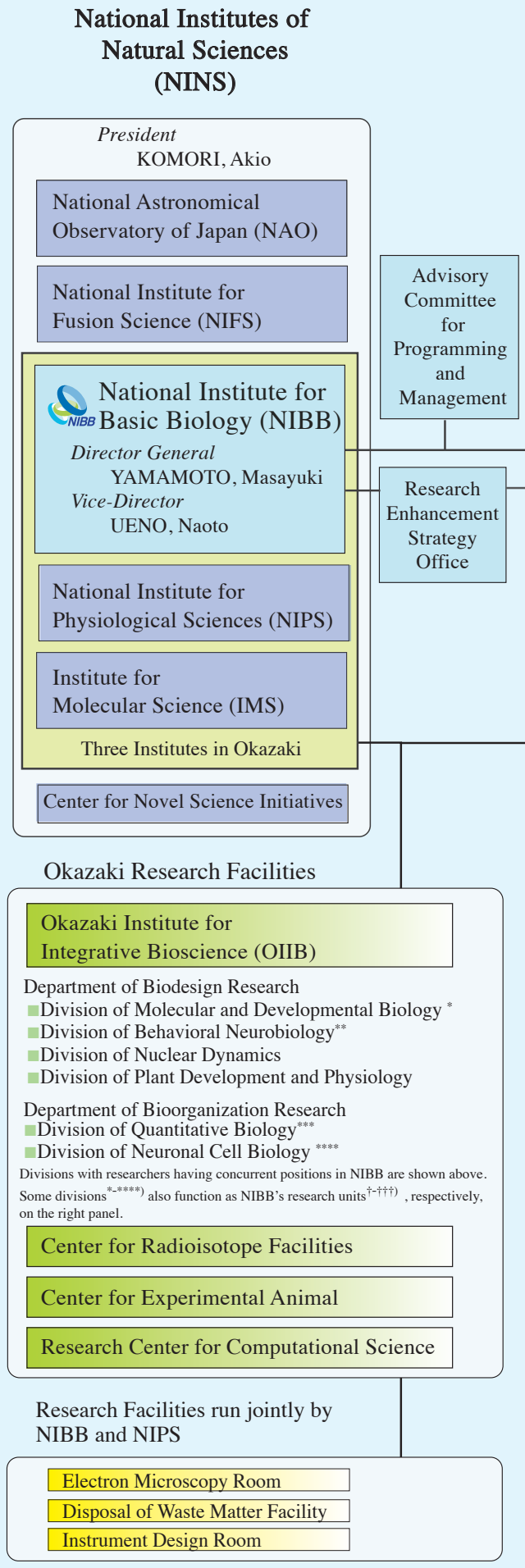
Policy, Decision Making, and Administration

The Director General oversees the operation of NIBB, assisted by the Advisory Committee for Programming and Management. The Advisory Committee, comprised of eleven professors within NIBB and ten leading biologists outside NIBB, advises the Director General on important matters such as planning joint research programs as well as on the scientific activities of NIBB. The Advisory Committee makes recommendations on the appointments of new Directors General, faculty appointments, NIBB's annual budget and future prospects.

The Research Enhancement Strategy Office, aimed at supporting researchers in order to improve NIBB's abilities as a collaborative research institution, was restructured in 2013 from the former Strategic Planning Department, Office of Public Relations, and Office of International Cooperation. The Office is made up of five groups (p. 90) and its activities are mainly carried out by URAs (University Research Administrators) in close collaboration with the NINS's Research Enhancement Promotion Headquarters.

Administration of NIBB is undertaken by the Okazaki Administration Office under the direct auspices of the Administration Office of NINS.

Organization



National Institute for Basic Biology (NIBB)

As of April 1, 2017

Research Units

- **Cell Biology**
  - Division of Cellular Dynamics
  - Division of Quantitative Biology†††
  - Division of Chromatin Regulation
  - Laboratory of Cell Responses
  - Laboratory of Neuronal Cell Biology††††
  - Laboratory of Stem Cell Biology
- **Developmental Biology**
  - Division of Morphogenesis
  - Division of Molecular and Developmental Biology †
  - Division of Embryology
  - Division of Germ Cell Biology
- **Neurobiology**
  - Division of Molecular Neurobiology
  - Division of Behavioral Neurobiology††
  - Laboratory of Neurophysiology
- **Evolutionary Biology and Biodiversity**
  - Division of Evolutionary Biology
  - Division of Symbiotic Systems
  - Division of Evolutionary Developmental Biology
  - Laboratory of Morphodiversity
  - Laboratory of Bioresources
  - Laboratory of Biological Diversity
- **Environmental Biology**
  - Division of Environmental Photobiology
  - Division of Seasonal Biology (Adjunct)
- **Theoretical Biology**
  - Laboratory of Genome Informatics
- **Imaging Science**
  - Laboratory for Spatiotemporal Regulations

Research Support Facilities

- **NIBB Core Research Facilities**
  - Functional Genomics Facility
  - Spectrography and Bioimaging Facility
  - Data Integration and Analysis Facility
- **NIBB BioResource Center**
  - Model Animal Research Facility
  - Model Plant Research Facility
  - Cell Biology Research Facility
- **NIBB Center of the Interuniversity Bio-Backup Project (IBBP Center)**
- **NIBB Center for Model Organism Development**

Research Support Section

- **Technical Division**

- Evaluation and Information Group
- Public Relations Group
- International Cooperation Group
- Collaborative Research Group
- Gender Equality Promotion Group

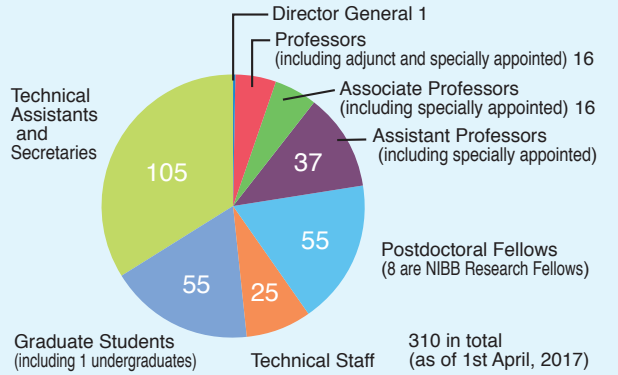
- **Okazaki Administration Office**

Research and Research Support

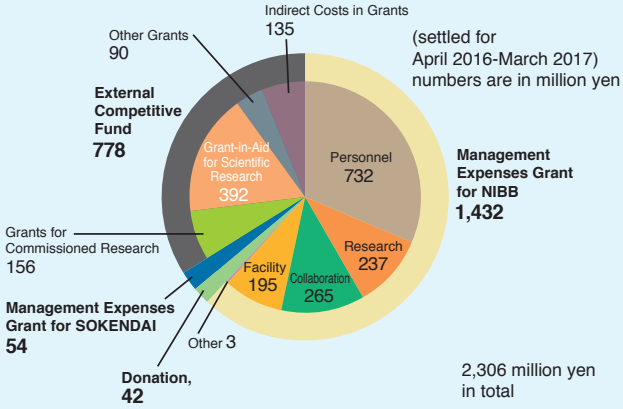
NIBB's research programs are conducted in research units and research support units. Each research unit is called either a division, led by a professor, or a laboratory, led by an associate professor or an assistant professor. Each research unit forms an independent project team. The NIBB Core Research Facilities and NIBB BioResource Center were launched in 2010 to support research in NIBB and also to act as an intellectual hub to promote collaboration among the researchers of NIBB and other institutions. The NIBB Center of the Inter-University Bio-Backup Project (IBBP Center) was founded in 2012 to prevent loss of invaluable biological resources. Projects for the development of bio-resource preservation technology are solicited by the IBBP center from 2013. The NIBB Center for Model Organism Development was founded in 2013 to promote development of new model organisms and research using them. The Technical Division manages the activities of the technical staff and helps to promote the activities of each research unit and to maintain the common resources of both the research support units of NIBB and the research facilities of the Okazaki campus. Center for Radioisotope Facilities are one of the latter and run by the technical staff of NIBB.

The Okazaki Institute for Integrative Bioscience was founded to conduct interdisciplinary molecular research on various biological phenomena and is shared by all three institutes in Okazaki. Some of the divisions also function as NIBB divisions.

Members in NIBB



Financial Configuration of NIBB



Members of the Advisory Committee for Programming and Management (as of April, 2017)

Non-NIBB members	AGATA, Kiyokazu*	Professor, Gakushuin University
	HIRAOKA, Yasushi*	Professor, Osaka University
	KOHCHI, Takayuki*	Professor, Kyoto University
	KOHSHIMA, Shiro	Professor, Kyoto University
	KUME, Shoen	Professor, Tokyo Institute of Technology
	NISHITANI, Kazuhiko ##	Professor, Tohoku University
	NOSE, Akinao	Professor, The University of Tokyo
	SASAKI, Hiroyuki*	Professor/ Senior Vice President, Kyushu University
	SUGIMOTO, Asako*	Professor, Tohoku University
	YAMAMOTO, Takashi	Professor, Hiroshima University
NIBB members	FUJIMORI, Toshihiko	Professor, National Institute for Basic Biology
	HASEBE, Mitsuyasu #	Professor, National Institute for Basic Biology
	HIGASHIJIMA, Shin-ichi	Professor, Okazaki Institute for Integrative Bioscience
	KAWAGUCHI, Masayoshi	Professor, National Institute for Basic Biology
	MINAGAWA, Jun	Professor, National Institute for Basic Biology
	NIIMI, Teruyuki	Professor, National Institute for Basic Biology
	NODA, Masaharu	Professor, National Institute for Basic Biology
	TAKADA, Shinji	Professor, Okazaki Institute for Integrative Bioscience
	UEDA, Takashi*	Professor, National Institute for Basic Biology
	UENO, Naoto	Professor, National Institute for Basic Biology
	YOSHIDA, Shosei	Professor, National Institute for Basic Biology

Chairperson
Vice-Chair
* new member from April 2017

GOALS OF THE NATIONAL INSTITUTE FOR BASIC BIOLOGY

The National Institute for Basic Biology (NIBB) sets five goals for its activities in pursuing the progress of biology. We contribute to the world-wide community of biologists through our efforts to accomplish these goals. This chapter briefly explains four of these goals. The last goal, the promotion of academic research, is accomplished through our research activities, which are introduced throughout this brochure.

Promotion of Collaborative Research

Collaborative Research Support

Research activities in collaboration with NIBB's divisions/laboratories using NIBB's facilities are solicited from external researchers. "Individual collaborative research projects" are the basic form of collaboration support which provide external researchers with travel and lodging expenses to visit NIBB's laboratories for collaborative research. "Priority collaborative research projects" are carried out as group research by internal and external researchers to develop pioneering research fields. "Collaborative research projects for model organism/technology development" and "Collaborative research projects for bioresource preservation technology development" are for developing and establishing new model organisms and new research technology. For these projects, research expenses in addition to travel expenses are provided. In 2016 two new projects, Collaborative research projects for integrative genomics and Collaborative research projects for integrative bioimaging, were initiated by reorganizing two former projects to facilitate more integrated use of the NIBB Core Research Facilities and to allow more intensive support through the planning, experimental, data analysis, and publication stages. Travel and lodging expenses are provided for these projects.

year	2013	2014	2015	2016
Priority collaborative research projects	2	1	2	2
Collaborative research projects for model organisms and technology development	4	2	3	2
Individual collaborative research projects	89	87	88	46
NIBB workshops	4	3	6	6
Collaborative experiments using the Large Spectrograph	15	12	10	10
Collaborative experiments using the DSLM	9	10	11	
Bioimage processing and analysis collaborative research projects			14	38*
Collaborative experiments using the next generation DNA sequencer	41	37	46	59**
Support for NIBB training courses	1	0	1	0
Collaborative research projects for bioresource preservation technology development	9	10	9	12
total	174	162	190	175

Collaborative Research Projects by Year
*renovated as "Collaborative research projects for integrative bioimaging"
**renovated as "Collaborative research projects for integrative genomics"

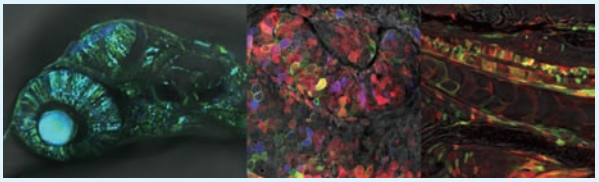
NIBB Core Research Facilities

The NIBB Core Research Facilities support research in NIBB and also act as an intellectual hub to promote collaboration between NIBB and other academic institutions. They consist of three facilities that are developing and providing state-of-the-art technologies through functional genomics, bioimaging and bioinformatics (p. 76).

The Functional Genomics Facility maintains a wide array of core research equipment, including next generation DNA sequencers. The facility is dedicated to fostering NIBB's collaborative research by providing these tools as well as expertise. The current focus is supporting functional genomics works that utilize mass spectrometers and DNA sequencers, holding training courses as one of these undertakings (p. 96). The Spectrography and Bioimaging Facility manages research tools, such as confocal microscopes, DSLM and the large spectrograph, and provides technical support and scientific advice to researchers. These two facilities hold specially appointed associate professors, an expert in each field, with a mission to manage each facility as well as conducting his own academic research. The Data Integration and Analysis Facility supports analysis of large-scale biological data, such as genomic sequence data, gene expression data, and imaging data. For this purpose, the facility maintains high-performance computers with large-capacity storage systems.

Bio-Resources

The National BioResource Project (NBRP) is a national project for the systematic accumulation, storage, and supply of nationally recognized bio-resources (experimental animals and plants, cells, DNA, and other genetic resources), which are widely used as materials in life science research. To promote this national project, NIBB has been appointed as a center for research on Medaka (*Oryzia latipes*) whose usefulness as a vertebrate model was first shown by Japanese researchers. The usability of Medaka as a research material in biology has drawn increasing attention since its full genome sequence recently became available. NIBB is also a sub-center for the NBRP's work with Japanese morning glory. The NIBB BioResource Center has equipment, facilities, and staff to maintain Medaka and Japanese morning glory safely, efficiently, and appropriately. The center also maintains other model organisms, such as mice, zebrafish, *Arabidopsis*, *Lotus japonicus*, and *Physcomitrella patens*, and provides technical support and advice for the appropriate use of these organisms (p. 83).



An example of a medaka strain in NBRP, Gaudi strain, in which individual cells in the brain and the retina are fluorescently labelled using Brainbow system.

NIBB Center of the Inter-University Bio-Backup Project (IBBP Center)

To prevent damage to important biological resources by natural disasters, NIBB established the IBBP Center in 2012 in collaboration with seven national universities for multiplicate preservation of genetic libraries and other invaluable

bioresources under cutting-edge research (p. 86).

International Cooperation and Outreach

Collaborative Programs with Overseas Institutes

NIBB takes a leading role in the collaborative research programs between the European Molecular Biology Laboratory (EMBL) and the National Institutes of Natural Sciences (NINS) and promotes personal and technological exchange through joint meetings, exchange between researchers and graduate students, and the introduction of experimental equipment.

NIBB formed an agreement with the Temasek Life Sciences Laboratory (TLL) of Singapore, Princeton University, and the Max Planck Institute for Plant Breeding Research (MPIPZ) to promote joint research projects, collaborative symposia, training courses and student exchange programs.

NIBB Conference

The NIBB Conferences are international conferences on hot topics in biology organized by NIBB's professors. Since the first conference in 1977 (the year of NIBB's foundation), NIBB Conferences have provided researchers in basic biology with valuable opportunities for international exchange. The 64th conference "Evolution of Seasonal Timers" was held in April, 2016 (p. 93).

International Practical Course

With the cooperation of researchers from Japan and abroad the NIBB international practical course is given at a laboratory specifically prepared for its use. The 9th course "Genetics and Imaging of Medaka and Zebrafish" was held jointly with TLL in August, 2016 at NIBB (P. 94). Graduate students and young researchers from various areas including Argentina, Korea, Singapore, Germany, India, Taiwan, UK, and Japan, were provided with training in state-of-the-art research techniques. International conferences and courses are managed by the International Cooperation Group of the Research Enhancement Strategy Office.

Outreach

NIBB's outreach activities aim to present cutting edge research results to the public via mass media through press releases or directly through the internet, such as web pages, Facebook, and Twitter. Our triannual open campus event was held in October, 2016 at which we welcomed more than 4,700 local citizens. NIBB also cooperates in the education of undergraduate and younger students through lectures and workshops. Outreach activities are mostly managed by the Public Relations Group of the Research Enhancement Strategy Office.

Development of New Fields of Biology

Bioimaging

NIBB aims to maximize the application of modern light microscopes and biophotonic probes for real time visualization of biological phenomena and to develop new imaging techniques. As part of our collaborative work with EMBL, NIBB introduced a DSLM, which is effective for the three-dimensional observation of living organisms, and has developed an improved model using two-photon optics (p. 73). The application of the adaptive optics to microscopy is under way in collaboration with the National Astronomical Observatory of Japan. The Advisory Committee on Bioimaging, comprised of leading researchers in the bioimaging field in Japan, is organized to formulate advice on NIBB's imaging research. The Bioimaging Forum provides an opportunity for researchers and company engineers to frankly discuss practical difficulties and needs regarding imaging. The 10th Forum " Pioneering Bioimaging in the New Era " was held in February, 2016 (p. 96). A training course in bioimage analysis was also held in 2016 (p. 97).

Okazaki Biology Conferences

NIBB holds Okazaki Biology Conferences (OBC) that, with the endorsement of the Union of Japanese Societies for Biological Science, aim to explore new research fields in biology and support the formation of international communities in these fields. Dozens of top-level researchers from Japan and abroad spend nearly one week together for intensive discussions seeking strategies for addressing future critical issues in biology. Past Conferences have promoted the formation of international researcher communities.

Cultivation of Future Researchers

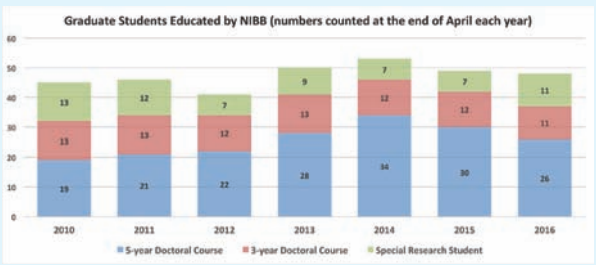
NIBB constitutes the Department of Basic Biology in the School of Life Science of the SOKENDAI (Graduate University for Advanced Studies). The department provides a five-year course for university graduates and a three-year doctoral course for graduate students with a master's degree.

Graduate students enrolled in other universities and institutions can apply to be special research students eligible to conduct research under the supervision of NIBB professors.

In both cases above, graduate students can receive financial support from NIBB based on the research assistant (RA) system from the beginning of the five-year course.

Due to the international collaboration with EMBL, graduate students are encouraged to attend PhD student symposia held at EMBL and provided an opportunity to give oral and poster presentations, at least once during their master's and doctoral program.

Students from Japan and abroad can also come to NIBB through our Internship Program. Internships give students an excellent way to build international connections while experiencing hands on research in a world class research institute (p. 98).



Graduate students educated by NIBB

Personnel changes in 2016*

Newly assigned in NIBB

Name	Position	Research Unit	Date
UEDA, Takashi	Professor	Division of Cellular Dynamics	April 1
AOKI, Kazuhiro	Professor	Division of Quantitative Biology	April 1
HIGASHIJIMA, Shin-ichi	Professor	Division of Behavioral Neurobiology	April 1
SOYANO, Takashi	Associate Professor	Division of Symbiotic Systems	April 1
EBINE, Kazuo	Assistant Professor	Division of Cellular Dynamics	April 1
KIMURA, Yukiko	Assistant Professor	Division of Behavioral Neurobiology	April 1
MORITA, Shinichi	NIBB Research Fellow	Division of Evolutionary Developmental Biology	April 1
ANDO, Toshiya	Assistant Professor	Division of Evolutionary Developmental Biology	July 1
TAKETSURU, Hiroaki	Specially Appointed Assistant Professor	IBBP Center	September 1
NARUSE, Kiyoshi	Specially Appointed Professor	Division of Germ Cell Biology	October 1
NAKAYAMA, Jun-ichi	Professor	Division of Chromatin Regulation	October 1
HAMADA, Kyoko	Assistant Professor	Division of Chromatin Regulation	October 1
KATAOKA, Kensuke	Assistant Professor	Division of Chromatin Regulation	December 1

Newly affiliated in other universities and institutes

Name	New Affiliation	Position	Date
NISHIMURA, Toshiya	Nagoya University	Assistant Professor	March 1
OGINO, Yukiko	Kyushu University	Associate Professor	April 1
MASAMIZU, Yoshito	University of Tokyo	Assistant Professor	April 1
IMAI, Akihiro	Hiroshima Institute of Technology	Assistant Professor	April 1
MATSUZAKI, Masanori	University of Tokyo	Professor	April 1
EBINA, Teppei	University of Tokyo	Assistant Professor	April 1
TANAKA, Minoru	Nagoya University	Professor	June 1
MIYAGAWA, Shinichi	Wakayama Medical University	Lecturer	June 1
SHIMMURA, Tsuyoshi	Tokyo University of Agriculture and Technology	Associate Professor (Tenure-Track)	November 16

* Changes in professors, associate and assistant professors, and NIBB research fellows are shown.

Awardees in 2016

Name	Position	Award
OHSUMI, Yoshinori	Professor Emeritus	The Nobel Prize in Physiology or Medicine, The Order of Culture (Bunka Kunsho), Dr. Paul Janssen Award for Biomedical Research, The Breakthrough Prize in Life Sciences
HASEBE, Mitsuyasu	Professor	Society of Evolutionary Studies, Japan's Eminent Evolutionalist Award
NAKAMURA, Yoshiaki	JSPS Researcher	The Society for Reproduction and Development's Outstanding Presentation Award

Prof. Yoshinori Ohsumi's laboratory at NIBB: Fermentation of yeast cell autophagy research

NIBB Professor Emeritus Yoshinori Ohsumi won the Nobel Prize in Physiology or Medicine for 2016 for his discoveries of the mechanisms for autophagy¶. Dr. Ohsumi presided over the Division of Molecular Cell Biology at NIBB for 13 years, from 1996 to 2009. It is a great honor for me, as an alumnus of his laboratory, to introduce to you a brief summary of the fruitful progress of Dr. Ohsumi's work.

In April 1996, Dr. Ohsumi came to NIBB from the University of Tokyo, with 13 yeast "*apg*" mutants, which have defects in autophagy*. At NIBB he made many advancements with his colleagues to investigate the molecular mechanisms of autophagy: cloning of yeast *ATG* genes, screening of additional *ATG* genes, biochemical and cell biological characterization of Atg proteins, and extensive autophagy studies in higher eukaryotes.

The main research achievements of Dr. Ohsumi's Laboratory are as follows:

Firstly, 18 Atg proteins were classified into 5 subgroups on the basis of biochemical analyses:

(1) Atg1 kinase and its regulators[1], recognizing nutrient starvation, an inducing signal of autophagy;

(2) Phosphatidylinositol 3-kinase complex[2], generating phosphatidylinositol 3-phosphate (PI-3P), an important material for autophagosome;

(3) Atg12 conjugation system[3];

(4) Atg8 conjugation system[4], both of which are ubiquitin-related systems, revealing that two intracellular protein degradation process, autophagy and ubiquitin-proteasome, employ a similar molecular device;

(5) Atg2-Atg18 complex and Atg9[5], PI-3P binding proteins and a membrane-integrating protein, respectively.

Secondly, fluorescence microscopy studies revealed that these Atg proteins hierarchically assemble together at "PAS" (pre-autophagosomal structure) to generate autophagosome[6]. Through these yeast studies, they established a schematic molecular model of autophagy[7]. And lastly they found that these findings derived from yeast studies are fundamentally conserved among another eukaryotes, especially mammals[8] and plants[9], to create a universal field of study of autophagy, an important protein degradation system.

(Yoshiaki Kamada)

References

1. Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., and Ohsumi, Y. (2000) Tor-Mediated Induction of Autophagy via an Apg1 Protein Kinase Complex. J. Cell Biol. *150*, 1507-1513.
2. Kihara, A., Noda, T., Ishihara, N., and Ohsumi, Y. (2001) Two Distinct Vps34 Phosphatidylinositol 3–Kinase Complexes Function in Autophagy and Carboxypeptidase Y Sorting in *Saccharomyces cerevisiae*. J. Cell Biol. *152*, 519-530.
3†. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M., and Ohsumi, Y. (1998) A protein conjugation system essential for autophagy. Nature *395*, 395-398.
4†. Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda T., and Ohsumi, Y. (2000) A ubiquitin-like system mediates protein lipidation. Nature *408*, 488-492.
5. Noda, T., Kim, J., Huang, W.-P., Baba, M., Tokunaga, C., Ohsumi, Y., and Klionsky, D. J. (2000) Apg9p/Cvt7p Is an Integral Membrane Protein Required for Transport Vesicle Formation in the Cvt and Autophagy Pathways. J. Cell Biol. *148*, 465-479.
6. Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T., and Ohsumi, Y. (2001) The pre-autophagosomal structure organized by concerted functions of *APG* genes is essential for autophagosome formation. The EMBO J. *20*, 5971-5981.
7. Nakatogawa, T., Suzuki, K., Kamada, Y., and Ohsumi, Y. (2009) Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nature Reviews Molecular Cell Biology *10*, 458-467.
8. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. The EMBO J. *19*, 5720-5728.
9. Hanaoka, H., Noda, T., Shirano, Y., Kato, T., Hayashi, H., Shibata, D., Tabata, S., and Ohsumi, Y. (2002) Leaf Senescence and Starvation-induced chlorosis are accelerated by the disruption of an Arabidopsis autophagy gene. Plant Physiol. *129*, 1181-1193.

¶ https://www.nobelprize.org/nobel_prizes/medicine/laureates/2016/

* Autophagy-related genes including "*APG*" genes were uniformly renamed as "*ATG*" genes in 2003.

†These two papers are selected as 'Key Publications' by the Nobel Committee.

Dr. Ohsumi's 60th birthday party (2005).

Dr. Ohsumi and Dr. Ohsumi's Lab members with Dr. Aaron Ciechanover, a ubiquitin researcher and a Nobel Laureate (2006).

Dr. Ohsumi and Dr. Ohsumi's Lab members posing "Ina Bauer" (2006).

Executive members of the NINS and NIBB staff gathering at the unveiling ceremony (2017).

Dr. Ohsumi visited NIBB for the unveiling ceremony of the Nobel prize monument, inspired by an image of the budding yeast (2017).

DIVISION OF CELLULAR DYNAMICS

Professor
UEDA, Takashi

Assistant Professor: *EBINE, Kazuo*
Postdoctoral Fellow: *EBINE, Kazuo**
SHIMADA, Takashi L.
TSUTSUI, Tomokazu
Visiting Graduate Student: *TAKEMOTO, Kodai*
KANAZAWA, Takehiko
MINAMINO, Naoki
NORIZUKI, Takuya
Secretary: *OKUBO, Masayo*

Membrane traffic between single membrane-bounded organelles plays integral roles in various cell activities in eukaryotic cells. Recent comparative genomics has indicated that membrane trafficking pathways are diversified among eukaryotic lineages, which is associated with lineage-specific acquisition of new trafficking pathways and secondary loss of preexisting trafficking routs. Our long-term goal is unraveling how plants have acquired their unique membrane trafficking systems during evolution, which will be achieved by comparative analyses using the model plant *Arabidopsis thaliana* and a model of liverworts, *Marchantia polymorpha*. We also aim to elucidate detailed molecular mechanisms and physiological functions of membrane trafficking in higher-ordered plant functions.

I. Diversification of membrane trafficking pathways associated with acquisition of novel machinery components

Although the basic framework of membrane trafficking is well conserved among eukaryotic lineages, recent comparative genomics has suggested that each lineage has acquired unique membrane trafficking pathways during evolution. RAB GTPases and SNARE proteins are evolutionarily conserved key regulators acting in tethering and/or fusion of membrane vesicles with target membranes. It has been proposed that lineage-specific diversification of these key factors is tightly associated with acquisition of lineage-specific membrane trafficking pathways, whose molecular basis remains unknown.

1-1. Characterization of RAB and SNARE proteins in the liverwort, *Marchantia polymorpha*

For information on the diversification of membrane trafficking pathways during land plant evolution, we systematically identified RAB GTPases and SNARE proteins in *Marchantia polymorpha*. Comparison of organization of these protein families with other plant lineages, followed by their functional analyses in *M. polymorpha*, indicated that diversification of membrane trafficking pathways in land plants has been achieved by 1) acquisition of novel machinery components, 2) relocating conserved machinery components to distinct trafficking events, and 3) secondary loss of conserved machinery components, during evolution.

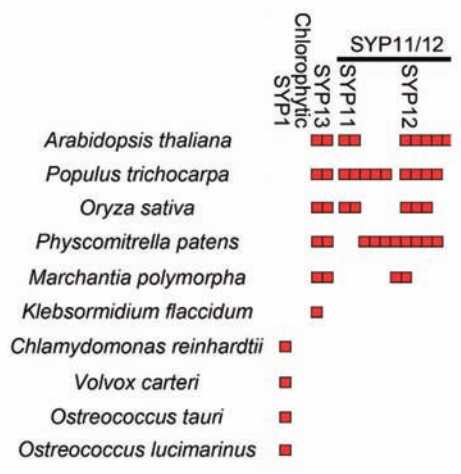


Figure 1. *SYP1* genes in the genomes of green plants. Genes are indicated as individual boxes. Numbers of *SYP1* genes increased during land plant evolution, suggesting functional diversification of SYP1 members in land plants.

1-2. Analysis of the liverwort-specific organelle, oil body

Through analyses of SNARE members in *M. polymorpha*, we found that a member of the SYP1 group is localized to the membrane of an organelle specific to liverworts, the oil body, whose origin and mechanisms of biogenesis remain unclear. We are currently analyzing the molecular function of the SYP1 member, as well as characterizing membrane trafficking pathways responsible for oil body biogenesis. We are also trying to isolate mutants defective in oil body functions by a forward-genetic approach.

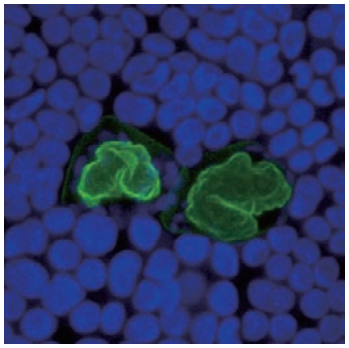


Figure 2. Oil bodies in a thallus of *M. polymorpha*, which are visualized by the YFP-tagged SYP1 member.

1-3. Mechanisms and dynamics of vacuolar transport

The vacuole is the largest organelle in plant cells, and occupies over 90 % of mature plant cells. The vacuole fulfills various functions in plant physiology and development, which include protein degradation, protein storage, and regulation of turgor pressure. To fulfill these vacuolar functions, a wide variety of vacuolar proteins and other components must be correctly transported to the vacuole, which is mediated by membrane trafficking. To understand molecular mecha-

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2016. The former title is indicated by an asterisk (*).

nisms of vacuolar transport in plants, we analyzed molecular functions of RAB5 and RAB7, and found that these proteins act sequentially in the vacuolar transport pathway in Arabidopsis cells. Furthermore, we also found that two additional vacuolar transport pathways, the RAB5-dependent but RAB7-independent pathway and the AP-3-dependent pathway operate in vacuolar transport in Arabidopsis. We are now exploring details of molecular mechanisms of these vacuolar transport pathways, especially focusing our interest on the RAB5-dependent but RAB7-independent pathway, because this trafficking pathway has not been described in non-plant systems.

SNARE proteins are also key molecules of vacuolar trafficking. Defective functions in vacuolar SNAREs affect both vacuolar transport and vacuolar morphology, which are distinctly regulated in plant cells. The *sgr3-1* (*shoot gravitropism3*) mutant was isolated as a mutant defective in shoot gravitropism, which resulted from a point mutation in SYP22/VAM3, one of the SNARE proteins residing on the vacuole and acting in vacuolar transport. Intriguingly, *sgr3-1* exhibits abnormal vacuolar morphology, although vacuolar transport is not markedly affected in this mutant. Therefore *sgr3-1* should be a good tool for dissecting functions of the vacuolar SNARE. We are exploring vacuolar dynamics regulated by SYP22/VAM3 by analyzing the effect of the *sgr3-1* mutation in a detailed manner.

II. Significance of membrane trafficking in higher-ordered plant functions

2-1. Analyses of functions of the plant-specific RAB GTPase ARA6 in stress responses in *Arabidopsis thaliana*

ARA6 is a plant-unique RAB GTPase, whose close homologs are found in only green plant lineages. To elucidate why only plants harbor the ARA6 members, we analyzed functional significance of ARA6 in biotic and abiotic stress responses. We found that ARA6 is recruited to the extrahaustorial membranes formed by the fungal pathogen causing powdery mildew and the oomycete causing downy mildew, suggesting modulation of host membrane trafficking by pathogenic microbes (Inada *et al*., 2016). We also found that proliferation of a pathogenic bacterium is repressed in the *ara6* mutant, which was due to an enhanced defense response mediated by elevated sugar concentration.

2-2. Membrane trafficking in plant gametogenesis

Gametogenesis in plants also involves membrane trafficking-mediated processes. We are now analyzing molecular mechanisms of gametogenesis in Arabidopsis and *M. polymorpha*, especially focusing our interests on secretory and degradative trafficking pathways during male gamete formation.

Publication List:

〔Original papers〕

- Ebine, K., Hirai, M., Sakaguchi, M., Yahata, K., Kaneko, O., and Saito-Nakano, Y. (2016). Plasmodium Rab5b is secreted to the cytoplasmic face of the tubovesicular network in infected red blood cells together with N-acylated adenylate kinase 2. Malar. J. *17*, 323.
- Inada, N., Betsuyaku, S., Shimada, T.L., Ebine, K., Ito, E., Kutsuna, N., Hasezawa, S., Takano, Y., Fukuda, H., Nakano, A., and Ueda, T. (2016). Modulation of plant RAB GTPase-mediated membrane trafficking pathway at the interface between plants and obligate biotrophic pathogens. Plant Cell Physiol. *57*, 1854-1864.
- Mbengue, M., Bourdais, G., Gervasi, F., Beck, M., Zhou, J., Spallek, T., Bartels, S., Boller, T., Ueda, T., Kuhn, H., and Robatzek, S. (2016). Clathrin-dependent endocytosis is required for immunity mediated by pattern recognition receptor kinases. Proc. Natl. Acad. Sci. USA *113*, 11034-11039.
- Sakurai, H.T., Inoue, T., Nakano, A., and Ueda, T. (2016). ENDOSOMAL RAB EFFECTOR WITH PX-DOMAIN, an interacting partner of RAB5 GTPases, regulates membrane trafficking to protein storage vacuoles in Arabidopsis. Plant Cell *28*, 1490-0503.
- Yoshinari, A., Fujimoto, M., Ueda, T., Inada, N., Naito, S., and Takano, J. (2016). DRP1-dependent endocytosis is essential for polar localization and boron-induced degradation of the borate transporter BOR1 in *Arabidopsis thaliana*. Plant Cell Physiol. *57*, 1985-2000.

〔Original papers (E-publication ahead of print)〕

- Akita, K., Kobayashi, M., Sato, M., Kutsuna, N., Ueda, T., Toyooka, K., Nagata, N., Hasezawa, S., and Higaki, T. Accumulation of fluorescent proteins derived from a *trans*-Golgi cisternal membrane marker and paramural bodies in interdigitated apoplastic space in *Arabidopsis* leaf epidermis. Protoplasma 2016 Mar 9.
- Cui, Y., Zhao, Q., Xie, H.T., Wong, W.S., Gao, C., Ding, Y., Tan, Y., Ueda, T., Zhang, Y., and Jiang, L. MON1/CCZ1-mediated Rab7 activation regulates tapetal programmed cell death and pollen development in *Arabidopsis*. Plant Physiol. 2016 Oct 31.

DIVISION OF QUANTITATIVE BIOLOGY

Professor
AOKI, Kazuhiro

Visiting Graduate Student: *KOMATSUBARA, Akira*
MIURA, Haruko
UDA, Youichi
MARYU, Gembu
Technical Assistant: *ITO, Reina*
Secretary: *ONODA, Kaori*

The Division of Quantitative Biology has been launched in April 2016. We are interested in the molecular mechanisms underlying cell-fate decision in mammalian cells.

Living cells act as input-output (I/O) units, in which environment and/or internal states are recognized on the cell surface and processed within a cell, leading to the adaptive response to these changes. This cellular information processing is mainly controlled by intracellular signal transduction, which is a series of chemical reactions, most commonly protein phosphorylation. Importantly, the dysregulation of signal transduction by gene mutation results in pathological diseases such as cancer.

The intracellular signaling pathway has been extensively studied over the last few decades, and most of its proteins and regulations have been identified, causing the perceived increase in the pathway's complexity. Computer-assisted simulation is one of the most promising approaches for the understanding of the signal transduction pathway as a system. Indeed, chemical and physical reactions comprising signal transduction can be described by ordinary differential equations, and solved numerically by computers. A number of simulation models of signaling pathways have, in fact, been reported to date. However, most of the kinetic parameters utilized for the simulation models were not measured experimentally, but rather were assumed by fitting the experimental data with the simulation model or simply determined arbitrarily. Consequently, there are substantial differences in the kinetic parameters between these studies, thereby making it difficult to evaluate these simulation models quantitatively.

To address these issues, we currently focus on the development of research tools enabling us to (1) visualize, (2) quantify, and (3) manipulate intracellular signaling pathways with fluorescence imaging techniques and computational approaches.

I. Visualization of cell signaling

With the advent of fluorescent proteins, it has been possible to visualize kinetic reactions at the single cell level. We are using the following two different approaches to unveil when and where the intracellular signaling event takes place:

1-1. Development of FRET-based biosensors

Förster (or fluorescence) resonance energy transfer (FRET) is a non-radiative process, in which excitation energy of donor fluorophore is transferred to a nearby acceptor fluorophore. By taking advantage of the principles of FRET, we have developed several biosensors that allowed us to detect kinase activity of PKA, ERK, Akt, JNK, PKC, S6K, and so on in a living cell with high temporal and spatial resolution (Figure 1).

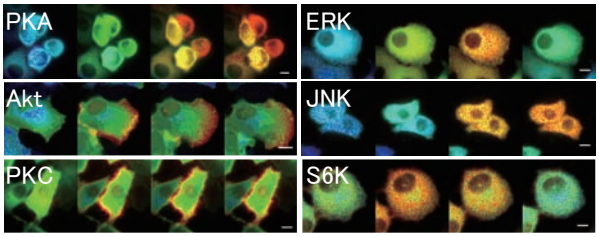


Figure 1. Visualization of PKA, ERK, Akt, JNK, PKC and S6K activities by FRET imaging. HeLa cells expressing FRET biosensors for the indicated kinases were stimulated with each ligand. Red and blue colors indicate activation and inactivation of the kinase, respectively.

1-2. Imaging of multiple kinase activities

Although FRET biosensors enable us to monitor cell signaling events, FRET imaging is not suited for multiplexed imaging, because these biosensors are authentically comprised of two different fluorophores in many cases. To circumvent this limitation, we are currently developing biosensors based on the principle of the kinase translocation reporter (KTR) system; the reporter is translocated from nucleus to cytoplasm when it is phosphorylated by its target kinase (Figure 2A). This approach yields several kinase reporters, e.g., ERK, Akt, p38, and JNK, and will provide clues as to how cells emerge all-or-none and irreversible cellular events such as cell cycle progression and apoptosis by using analog and continuous kinase signals as an input (Figure 2B).

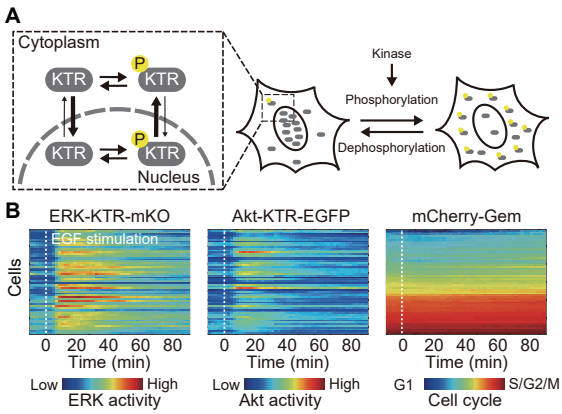


Figure 2. Multiplexed imaging of ERK and Akt activities and cell cycle state. A, Schematic representation of the principle of KTR system. B, Shown here are heat maps of ERK activity (left), Akt activity (middle), and cell cycle (right) obtained by KTR systems.

II. Quantification of cell signaling

Classically, kinetic parameters such as protein concentration and dissociation constant have been measured by *in vitro* biochemical analyses. However, some kinetic parameters might significantly differ between *in vitro* and *in vivo*. For instance, the Kd values measured *in vivo* were higher than

the *in vitro* Kd values by an order of 1 or 2. Therefore, it is critical to measure kinetic parameters in living cells. To this end, we combine CRISPR/Cas9-mediated genome editing with fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS) to quantitatively determine the endogenous protein concentration and the dissociation constant (Figure 3). These parameters will be applied to the computer simulation to predict the dynamics of singling pathways.

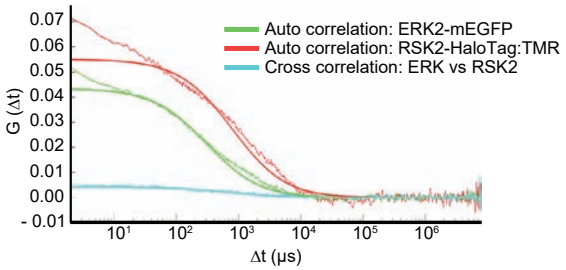


Figure 3. Auto- and cross-correlation functions of ERK2-mEGFP and RSK2-HaloTag. *mEGFP* and *HaloTag* genes were knocked-in at the site of 3' *ERK2* and *RSK2* genes, respectively. These auto- and cross-correlation functions were obtained by FCS and FCCS, providing endogenous concentration of ERK2 and RSK2 and the dissociation constant of ERK2-RSK2 binding.

III. Manipulation of cell signaling

Optogenetics is a powerful tool for the control of the intracellular signaling pathway with high spatial and temporal precision. Our laboratory focuses on the light-induced dimerization (LID) system with plant cryptochrome 2 (CRY2) and phytochrome B (PhyB), in which blue- and red-light induce heterodimerization to their binding partners, respectively. By using these systems, we attempt to reconstitute the spatial and temporal pattern of cell signaling in order to directly validate the physiological meaning of the dynamics of cell signaling that were observed by time-lapse fluorescence imaging.

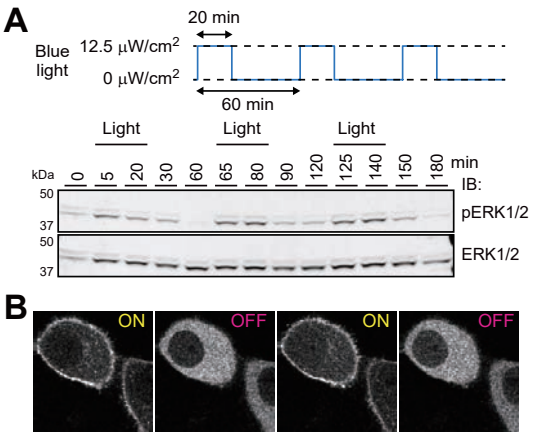


Figure 4. Optogenetic manipulation of intracellular signaling. A, Blue light-induced repetitive ERK activation with CRY2 system. B, Red and infrared light-induced shuttling of phytochrome interacting factor (PIF) -EGFP between cytoplasm and the plasma membrane.

Publication List:

〔Original papers〕

- Inaba, K., Oda, K., Aoki, K., Sone, K., Ikeda, Y., Miyasaka, A., Kashiyama, T., Fukuda, T., Makii, C., Arimoto, T., Wada-Hiraike, O., Kawana, K., Yano, T., Osuga, Y., and Fujii, T. (2016). Synergistic antitumor effects of combination of PI3K/mTOR and MEK inhibition (SAR245409 and pimasertib) in mucinous ovarian carcinoma cells by fluorescence resonance energy transfer imaging. Oncotarget *7*, 29577-29591.
- Kamezaki, A., Sato, F., Aoki, K., Asakawa, K., Kawakami, K., Matsuzaki, F., and Sehara-Fujiwara, A. (2016). Visualization of Neuregulin 1 ectodomain shedding reveals its local processing in vitro and in vivo. Sci. Rep. *6*, 28873.
- Maryu, G., Matsuda, M., and Aoki, K. (2016). Multiplexed fluorescence imaging of ERK and Akt activities and cell-cycle progression. Cell Struct. Funct. *41*, 81-92.
- Yamao, M., Aoki, K., Yukinawa, N., Ishii, S., Matsuda, M., and Naoki, H. (2016). Two new FRET imaging measures: linearly proportional to and highly contrasting the fraction of active molecules. PLoS One *11*, e0164254.

DIVISION OF CHROMATIN REGULATION

Professor
NAKAYAMA, Jun-ichi

Assistant Professor:	*HAMADA, Kyoko*
	KATAOKA, Kensuke
Postdoctoral Fellow:	*KAWAGUCHI, Takayuki*
	*HAMADA, Kyoko**
Visiting Undergraduate:	*OKI, Koichi*
Technical Assistant:	*TANAKA, Mayo*

The stable inheritance of gene expression or repression state is essential to cellular differentiation and development, and an epigenetic cellular-memory system derived from higher-order chromatin structure plays a fundamental role in this process. The assembly of higher-order chromatin structure has been linked to the covalent modifications of histone tails, chromatin-associated proteins, and non-coding RNAs. The exact means by which such chromatin-based epigenetic information is established and faithfully maintained across cell divisions and throughout development, however, remains incompletely understood. To try to gain a better understanding of the molecular mechanisms underlying chromatin-based epigenetic phenomena, our lab uses fission yeast, *Schizosaccharomyces pombe*, and ciliate *Tetrahymena* as model organisms for studying the molecular mechanisms of higher-order chromatin assembly. We are also attempting to determine the cellular functions of chromatin modifying factors in developing a better understanding of complicated epigenetic phenomena in higher eukaryotic cells.

I. Establishment and maintenance of higher-order chromatin structure

In eukaryotic cells, the assembly of higher-order chromatin structure, known as heterochromatin, plays an important role in diverse chromosomal processes. We have previously shown that the specific methyl modification on lysine 9 of histone H3 (H3K9me) and the binding of the chromodomain (CD) proteins such as HP1 to methylated histones are essential to the assembly of higher-order chromatin structure. In fission yeast, the H3K9me is catalyzed by the methyltransferase Clr4. It was previously shown that Clr4 forms a complex called CLRC, which includes Rik1, Raf1, Raf2, and Cul4. Since Cul4 is one of the ubiquitin ligases, and Rik1 and Raf1 show sequence similarities to mammalian DDB1 and DCAF, respectively, it is hypothesized that CLRC has E3 ubiquitin ligase activity. However, CLRC's physiological targets remained unclear. We demonstrated that affinity purified CLRC preferentially ubiquitylates histone H3. Interestingly, H3 ubiquitylation was found to promote H3K9 methylation by Clr4. These results suggested that histone ubiquitylation plays a crucial role in maintaining H3K9me. We will further characterize the crosstalk between histone methylation and ubiquitylation by defining how ubiquitylation promotes Clr4 and which domain(s) of Clr4 is responsible for binding ubiquitylated histone H3.

II. Regulation of chromodomain proteins in assembling higher-order chromatin structure

The chromodomain (CD) is a conserved sequence motif present in chromosomal proteins and functions as a binding module that targets methylated histone tails. Heterochromatin protein 1 (HP1) is an evolutionarily conserved chromodomain protein that binds to lysine H3K9me, a hallmark of heterochromatin. We previously showed that mammalian HP1α is phosphorylated at its N-terminal serine residues and that this phosphorylation contributes to HP1α's specific binding to H3K9me nucleosomes and is essential for its proper targeting to heterochromatin. We further demonstrated that Swi6, one of fission yeast HP1 homologues, is also subjected to phosphorylation at its N-terminal conserved region, and also that this region is critical for Swi6's silencing function (Figure 1). We will further characterize the mechanisms how Swi6's N-terminal region contributes to its silencing function by identifying factors that specifically or differentially interact with the Swi6's N-terminal region.

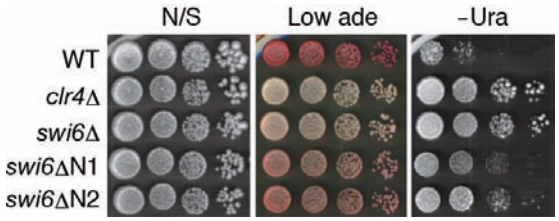


Figure 1. Swi6 N-terminal region is required for its silencing function. A 10-fold serially diluted culture of indicated strain was spotted onto nonselective medium (N/S), low adenine medium (Low Ade), or medium lacking uracil (–Ura).

III. Characterizing nucleic acid-binding activities associated with chromatin proteins

The CD's best-characterized targets are methylated histone tails. Several lines of evidence suggest that the CD, in addition to targeting methylated histones, also binds to DNA and RNA. Although both the nucleic acid-binding and methylated histone-binding activities of CDs are known to be important for CD protein functions in several biological processes, the physical and functional relationships between these two activities are poorly understood. We previously showed that fission yeast Chp1, a CD protein functioning in the RNA silencing pathway, possesses unique nucleic acid-binding activities that are essential for heterochromatic gene silencing. To obtain further evidence that nucleic acid-binding activity associated with CD plays an important role in their chromatin targeting, we focused on mammalian CD proteins and showed that their nucleic acid-binding activity is critical for their nucleosome binding or heterochromatin assembly. We will further characterize the functional role of the CD's nucleic acid-binding activity and also try to identify chromatin factors that specifically interact with non-coding RNAs.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2016. The former title is indicated by an asterisk (*).

IV. Dynamic chromatin reorganization during sexual reproduction of *Tetrahymena*

Tetrahymena, a unicellular protozoan, shows nuclear dimorphism. They contain the transcriptionally silent, germline micronucleus (MIC) and the transcriptionally active, somatic macronucleus (MAC) in a single cell (Figure 2). During sexual reproduction, the newly synthesized MAC removes the dispensable internal eliminated sequences (IESs) from the genome and adds telomeres at the ends of fragmented chromosomes. The IES elimination requires RNA interference (RNAi)-related pathway and posttranslational histone modifications. The methylation of histone H3 lysine 9 and/or lysine 27 catalyzed by Ezl1p acts as binding site for chromodomain proteins such as Pdd1 to form heterochromatin-like structure. Although the RNAi-related pathway to target IES regions is extensively studied, the underlying molecular mechanisms for heterochromatin assembly remain poorly understood. We will focus on uncharacterized heterochromatin proteins and examine their roles in dynamic change of chromatin structure. In addition, we will try to identify factors that are involved in chromosome fragmentation and *de novo* telomere addition during MAC development.

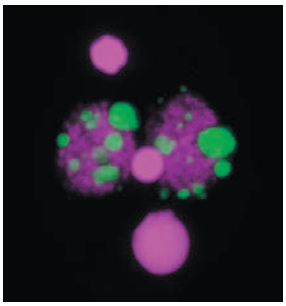


Figure 2. Developing macronuclei (MACs) during sexual reproduction of *Tetrahymena*. The cells at 14 hours post-mixing were stained with anti-Pdd1 (green) and DAPI (magenta). Pdd1 was detected exclusively in the new MACs.

Publication List:

〔Original papers〕

- Kamata, K., Shinmyozu, K., Nakayama, J., Hatashita, M., Uchida, H., and Oki, M. (2016). Four domains of Ada1 form a heterochromatin boundary through different mechanisms. Genes Cells. *21*, 1125-1136.
- Mitsumori, R., Shinmyozu, K., Nakayama, J., Uchida, H., and Oki, M. (2016). Gic1 is a novel heterochromatin boundary protein *in vivo*. Genes Genet. Syst. *91*, 151-159.
- Shimojo, H., Kawaguchi, A., Oda, T., Hashiguchi, N., Omori, S., Moritsugu, K., Kidera, A., Hiragami-Hamada, K., Nakayama, J., Sato, M., and Nishimura, Y. (2016). Extended string-like binding of the phosphorylated HP1α N-terminal tail to the lysine 9-methylated histone H3 tail. Sci. Rep. *6*, 22527.

LABORATORY OF CELL RESPONSES

Director General
YAMAMOTO, Masayuki

Specially Appointed Associate Professor
YAMASHITA, Akira

Postdoctoral Fellow: *OTSUBO, Yoko*
SHICHINO, Yuichi

Secretary: *SAKAGAMI, Mari*

Cells sense the environment around them, for example the amount of nutrients and hormones, as well as the temperature and pressure, and decide what kind of activities to undertake using this information. Germ cells, which produce sperm and eggs, begin halving their number of chromosomes during a special kind of cell division called meiosis, in response to the ambient conditions. In our laboratory, we use the fission yeast *Schizosaccharomyces pombe*, the simplest organism that performs meiosis, to research the mechanism by which cells switch from mitosis, the kind of cell division that divides cells equally to create two identical cells, to meiosis, which is essential for bringing forth genetically diverse progeny.

I. Signaling pathways that regulate the onset of sexual differentiation

We have been trying to elucidate how fission yeast cells switch their mode of cell cycle from mitotic to meiotic. We focus on a highly conserved kinase, namely Target of rapamycin (TOR) kinase, which plays key roles in the recognition of nutrition and the onset of sexual differentiation in fission yeast. TOR kinase forms two types of complexes, namely TORC1 and TORC2. TORC1 contains Tor2 as its catalytic subunit and is essential to suppress sexual differentiation in the presence of nitrogen. TORC2 contains Tor1 and, in contrast to TORC1, is required for onset of sexual differentiation under nitrogen starvation (Figure 1).

Temperature-sensitive *tor2* mutants initiate sexual differentiation on rich medium at the restrictive temperature. To gain insights into the TORC1 signaling pathway, we have isolated mutants that initiate sexual differentiation ectopically under nutrient-rich conditions. We are currently characterizing these mutants.

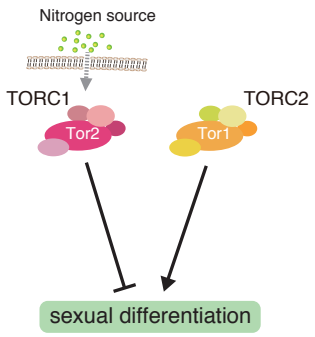


Figure 1. The two TOR complex pathways in *S. pombe*. TORC1, containing Tor2, and TORC2, containing Tor1, regulate sexual differentiation oppositely. TORC1 suppresses sexual differentiation in the presence of ample nitrogen.

II. The molecular mechanisms that establish the meiosis-specific transcription profile

Expression of hundreds of genes are upregulated during meiosis. We have shown that specific control of the stability of meiotic transcripts, which is orchestrated by the interplay between RNA-binding proteins and a long non-coding RNA, contributes to the meiosis-specific gene expression in fission yeast. Understanding precise mechanisms of this control will shed light on the regulation of timely gene expression during meiosis.

A YTH-family RNA-binding protein Mmi1 plays a crucial role in the selective elimination system of meiosis-specific transcripts during the mitotic cell cycle. Mmi1 recognizes a region termed DSR (Determinant of Selective Removal) in meiotic transcripts, which is enriched with repeats of hexanucleotide motifs. Meiotic transcripts bound to Mmi1 are degraded by the RNA-degradation nuclear exosome machinery. Mmi1 also induces formation of facultative heterochromatin at a subset of its target genes.

During meiosis, a meiosis-specific nuclear body, called Mei2 dot, blocks the Mmi1-mediated elimination system. The Mei2 dot is composed of the RNA-binding protein Mei2 and a long non-coding RNA species termed meiRNA. Mei2 physically interacts with meiRNA and forms the dot structure at the chromosomal *sme2* locus, which encodes meiRNA. The Mei2 dot lures Mmi1 through numerous copies of the DSR motif on meiRNA and inhibits its function, so that meiotic transcripts harboring DSR are stably expressed (Figure 2).

We have demonstrated that Mmi1 prevents untimely translation of meiotic transcripts by regulating their localization. Multilayered suppression of meiotic genes by Mmi1 is vital for mitotic growth.

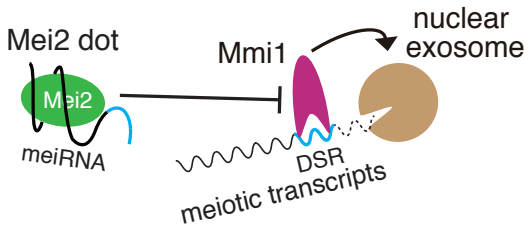


Figure 2. Selective elimination of meiosis-specific transcripts by the Mmi1/DSR system. Mmi1 binds to DSR in meiotic transcripts and induces their degradation by the nuclear exosome during the mitotic cell cycle. In meiotic cells, the Mei2 dot, composed of Mei2 and meiRNA, sequesters and inhibits Mmi1, so that DSR-harboring meiotic transcripts escape from Mmi1-mediated selective elimination.

III. Regulation of nuclear oscillation driven by cytoplasmic dynein during meiotic prophase

During meiotic prophase in fission yeast, the nucleus migrates back and forth between two poles of the cell. This oscillatory nuclear movement is called 'horse-tail' movement due to its characteristic shape and motion (Figure 3). Horse-tail nuclear movement enhances pairing of homologous chromosomes and facilitates meiotic recombination. Horse-tail movement is driven by cytoplasmic dynein, which forms a huge minus-end-directed microtubule motor complex, and dynactin, which is a protein complex that is required for most dynein-mediated cellular activities. Cytoplasmic dynein that is anchored to the cell cortex generates a pulling force on the microtubule emanating from the leading edge of the nucleus. This dynein-mediated pulling is the major contributor to horse-tail movement. Cortical anchoring of dynein is crucial for the generation of horse-tail movement.

We identified novel subunits of dynactin, and showed that dynein-related cortical factor, Num1, cooperates with dynactin to establish dynein anchoring at the cell cortex. We are now studying how the oscillatory movement of the nucleus is accomplished by combining computational simulations with live cell imaging (Figure 4).

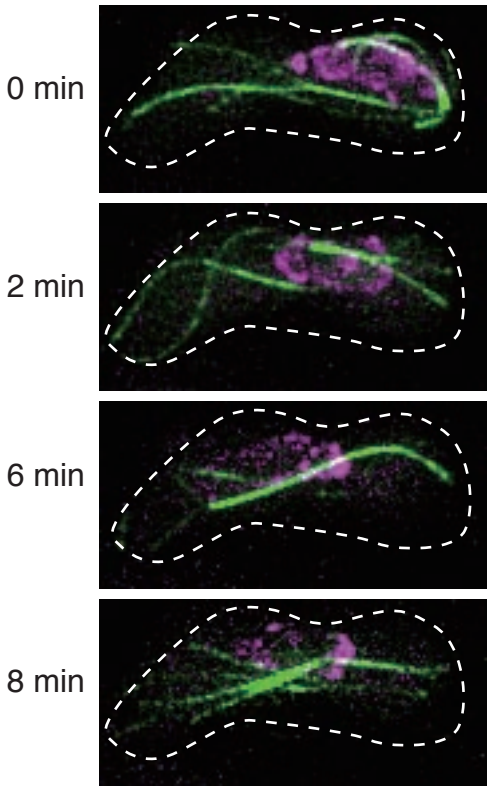


Figure 3. Horse-tail nuclear movement during meiotic prophase in *S. pombe*. Time-lapse images of nuclear membrane (Cut11, magenta) and microtubules (Atb2, green) in the wild-type strain. The cellular contour is shown by the dotted line.

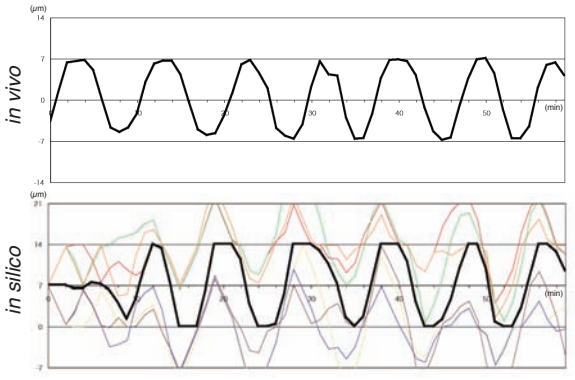


Figure 4. Tracks of the leading edge of the nucleus during horse-tail nuclear movement in a living cell and in a simulation model. Colored lines in the model indicate tracks of microtubule plus ends.

Publication List:

〔Review articles〕

- Yamashita, A., Shichino, Y., and Yamamoto, M. (2016). The long non-coding RNA world in yeasts. Biochim. Biophys. Acta Gene Regul. Mech. *1859*, 147-154.
- Yamashita, A., Sakuno, T., Watanabe, Y., and Yamamoto, M. (2016). Analysis of *Schizosaccharomyces pombe* Meiosis. Fission Yeast, A Laboratory Manual, 251-259.
- Yamashita, A., Sakuno, T., Watanabe, Y., and Yamamoto, M. (2016). Live imaging of chromosome segregation during meiosis in the fission yeast *Schizosaccharomyces pombe*. Fission Yeast, A Laboratory Manual, 260-263.
- Yamashita, A., Sakuno, T., Watanabe, Y., and Yamamoto, M. (2016). Synchronous induction of meiosis in the fission yeast *Schizosaccharomyces pombe*. Fission Yeast, A Laboratory Manual, 264-267.
- Yamashita, A., Sakuno, T., Watanabe, Y., and Yamamoto, M. (2016). A simple method to induce meiosis and sporulation semisynchronously in the fission yeast *Schizosaccharomyces pombe*. Fission Yeast, A Laboratory Manual, 268-270.

LABORATORY OF NEURONAL CELL BIOLOGY

Associate Professor
SHIINA, Nobuyuki

Assistant Professor: *NAKAYAMA, Kei*
SOKENDAI Graduate Student: *OHASHI, Rie*
KATAYAMA, Kaori
YAMASHITA, Akira
Technical Assistant: *MATSUDA, Chisato*

The transport of specific mRNAs and local control of translation in neuronal dendrites represent an important gene expression system that provides dendritic protein synthesis at just the right time and place. It is widely accepted that this system controls the location at which neurites will connect to each other, thereby forming neural networks. Our main interest is to understand the mechanisms and roles of mRNA transport and local translation in neuronal dendrites.

Specific mRNAs are recruited into "RNA granules" and transported to dendrites. RNA granules are membrane-less macromolecular assemblies composed mainly of mRNAs, ribosomes and RNA-binding proteins, and mediate the transport and local translation of their mRNA cargoes in response to synaptic stimulation (Figure 1). We are researching RNA granule factors regulating mRNA transport and local translation, their target mRNAs, and the mechanisms of localized protein synthesis using mice in order to better understand their relation to the formation of synapses and neural networks, memory, learning, and behavior.

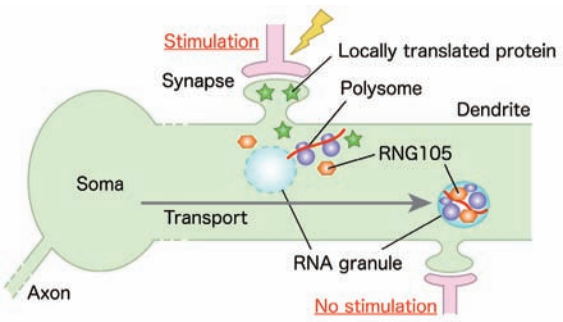


Figure 1. A model for local translation in neuronal dendrites. Specific mRNAs are recruited into RNA granules and transported to dendrites. Translation of the mRNAs is induced locally upon synaptic stimulation, which modifies local postsynapses to regulate synaptic connection and network formation.

I. Autism spectrum disorder (ASD)-like behaviors in RNG105 heterozygous mice

We previously identified RNA granule protein 105 (RNG105, also known as Caprin1), an RNA-binding protein, as a component of RNA granules. RNG105 is responsible for mRNA transport to dendrites, which is required for the encoded proteins to be translated and function in dendrites for proper networking of neurons.

A recent study reported that a heterozygous mutation in the *Rng105/Caprin1* gene was found in an autism spectrum disorder (ASD) patient, but it remained unclear whether there is a causal relationship between RNG105 deficiency and ASD. We then subjected *Rng105*$^{+/-}$ mice to a comprehensive behavioral test battery, and revealed the influence of RNG105 deficiency on mouse behavior. *Rng105*$^{+/-}$ mice exhibited a reduced sociality in a home cage and a weak preference for novel mice. Consistently, the *Rng105*$^{+/-}$ mice also showed a weak preference for novel objects and novel place patterns. Furthermore, although the *Rng105*$^{+/-}$ mice exhibited normal memory acquisition in spatial reference tasks, they tended to have relative difficulty in reversal learning in the tasks. These findings suggest that the RNG105 heterozygous knockout leads to a reduction in sociality, response to novelty and flexibility in learning, which are implicated in ASD-like behavior.

II. Long-term memory deficits in RNG105 conditional knockout (cKO) mice

Although learning and memory, except for reversal learning, were normal in mice with a moderate deficiency of RNG105 (*Rng105*$^{+/-}$), learning and memory were remarkably impaired in mice with severe deficiency of RNG105: RNG105 conditional knockout (cKO) postnatally in the brain region caused reduced contextual and spatial long-term memories.

For example, RNG105 cKO mice showed severe deficits in the Morris water maze, a spatial long-term memory task in which a mouse is placed in a circular pool and learns the location of a hidden platform that allows the mouse to escape from water. Repeated trials enabled control mice to learn the platform location and escape on the platform faster than before the trials (Figure 2A). In contrast, the escape latency of RNG105 cKO mice did not shorten at all over the trials (Figure 2A). Following the last trial, a probe test was conducted: the platform was removed from the pool and the swimming path of the mice was visualized by tracking the mice. Control mice intensively searched around the target

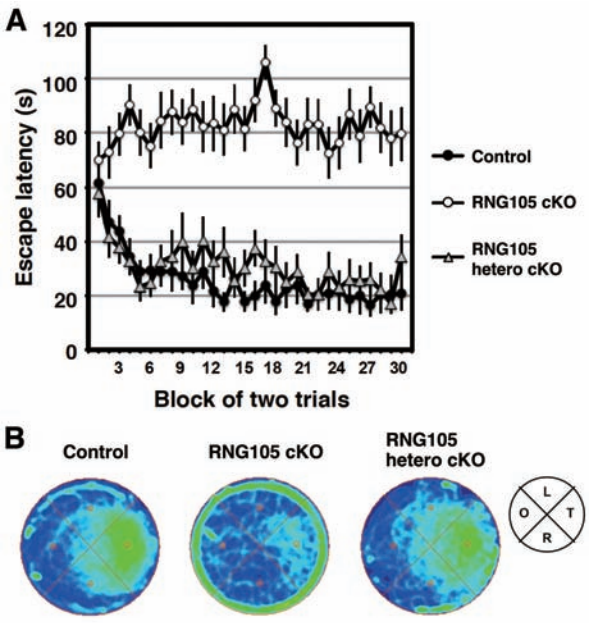


Figure 2. Spatial memory deficits of RNG105 cKO mice in Morris water maze. (A) Escape latency during the acquisition phase of the Morris water maze. (B) Spatial histograms of the mice's swimming paths during the probe trial of the Morris water maze. T indicates the target quadrant.

place where the removed platform had existed (Figure 2B). In contrast, RNG105 cKO mice showed a circular swimming path along the wall of the pool and reduced the search time around the target place (Figure 2B).

III. Reduced structural synaptic plasticity in RNG105 cKO mice

Memory formation is generally correlated with structural plasticity of postsynapses (spines) on dendrites, i.e., stimulation-induced long-lasting increase in the size of spines. Therefore, we measured the stimulation-dependent changes in spine size in RNG105 cKO cultured neurons. In response to synaptic stimulation by glutamate uncaging, spines near the uncaging locus increased in volume ~2.5-fold and sustained the increased state at least for 60 min in control neurons (Figures 3A and 3B). In contrast, in RNG105 cKO neurons, spine volume during the sustained phase was significantly reduced (Figure 3B). These results suggested that structural plasticity of spines was reduced by RNG105 deficiency.

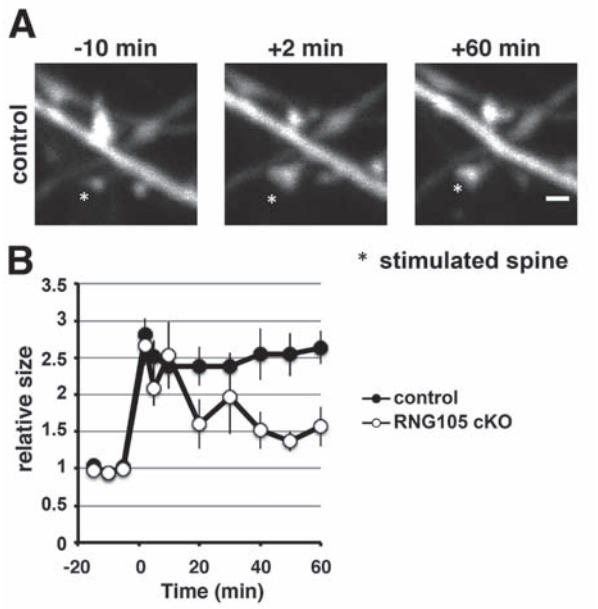


Figure 3. Reduced structural plasticity of spines in RNG105 cKO neurons. (A) Representative spine images before and after synaptic stimulation by glutamate uncaging. Scale bar, 1 μm. (B) Time-lapse graph of the effect of synaptic stimulation on spine size.

IV. Reduced localization of mRNAs in dendrites of RNG105 cKO neurons

To investigate the mechanism of how RNG105 cKO affects synaptic plasticity and long-term memory, we comprehensively identified mRNAs whose dendritic localization was reduced in RNG105 cKO neurons. The soma and dendrites of neurons were isolated by microdissection of the hippocampal stratum pyramidale (SP) layer and stratum radiatum (SR) layer, respectively, and then subjected to RNA-seq analysis using next-generation sequencing. By comparing mRNAs from the soma and dendrites, we identified 1,122 dendritically enriched mRNAs and 2,106 somatically enriched mRNAs. Enrichment of most of the dendritic mRNAs in dendrites was significantly reduced in RNG105 cKO neurons, which suggested that RNG105 is responsible for the dendritic localization of many different mRNAs.

Gene ontology enrichment analysis of the identified mRNAs revealed that categories in which significantly large number of mRNAs were enriched were quite different between somatic and dendritic mRNAs. Major categories for dendritic mRNAs were “Regulation of Arf protein signal transduction” which included GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) of small G protein Arf and “Structural constituent of ribosomes” which included ribosomal subunit proteins. Arf is known to regulate membrane trafficking between the cell surface and endosomes through controlling endocytosis and exocytosis, which includes glutamate receptor (AMPA receptor) surface expression in neurons. Arf is also known as an important regulator of actin cytoskeleton dynamics and dendritic spine formation via Rac1 activation. “Regulation of small GTPase-mediated signal transduction” was also a category in which dendritic mRNAs were enriched, which included regulators of small G proteins such as Ras, Rho and Rac, known to be involved in synaptic and actin regulation. Dendritic mRNAs and gene ontology categories identified in this study provide insight into underlying mechanisms for dendritic mRNA localization-dependent long-term synaptic plasticity and memory, which will be addressed in the future.

Publication List:

〔Original paper〕

- Ohashi, R., Takao, K., Miyakawa, T., and Shiina, N. (2016). Comprehensive behavioral analysis of RNG105 (Caprin1) heterozygous mice: Reduced social interaction and attenuated response to novelty. Sci. Rep. *6*, 20775.

LABORATORY OF STEM CELL BIOLOGY

Associate Professor
TSUBOUCHI, Tomomi

NIBB Research Fellow: *KAMIKAWA, Yasunao*
Postdoctoral Fellow: *TSUBOUCHI, Hideo*
Technical Assistant: *YASUI, Naomi*

DNA is constantly damaged from both endogenous and exogenous sources. One of the most important challenges for all living organisms is to prevent genome instability that can lead to malfunction of a cell. Our group is interested in the strategies through which cells protect themselves from alterations in the genome. To date, much information is gained from various model organisms and tissue culture cells, and we are beginning to learn that the choice of genome-maintenance strategies taken by a cell depends on the cell type, cell cycle- and developmental stages. In the Laboratory of Stem Cell Biology, we are currently focusing our attention on the genome maintenance mechanisms of the embryonic stem cells, and their roles during differentiation and reprogramming processes.

I. Self-renewal of Embryonic Stem Cells and Their Genome-Maintenance Mechanisms

Embryonic stem (ES) cells are derived from the blastocyst stage of embryonic development, and are capable of differentiating into all cell types that compose our body (i.e., ES cells are "pluripotent"). Pluripotent cells exist only transiently and are lost as development proceeds. On the other hand, ES cells are capable of proliferating indefinitely when given an appropriate culturing condition. Curiously, ES cells proliferate with truncated gap phases while S (DNA replication) and M (mitosis) phases take as much time as other cell types. ES cells also appear to lack some of the mechanisms that ensure genome integrity (i.e., checkpoint mechanisms), the significance of which remains a mystery.

To date, studies on cell cycle regulation in ES cells have not been straightforward compared to that of other cell types, as many commonly used cell-synchronization protocols are ineffective for ES cells. We have now established several protocols to synchronize ES cells (Tsubouchi et al., Cell, 2013; unpublished), which allowed us to investigate specific stages of the ES cell cycle. In 2016, we have closely investigated DNA replication and mitosis in ES cells and have found a few features that are unique to ES cells. We are currently aiming to address how such differences are interlinked with pluripotency by carrying out side-by-side analyses between ES cells and differentiated populations.

II. Genome Instability during Nuclear Reprogramming

In order to gain a deeper understanding of the relationship between the choice of genome maintenance mechanisms and pluripotency, we are investigating the behavior of factors involved in genome maintenance mechanisms during nuclear reprogramming towards pluripotency.

Specifically, we take advantage of the cell-to-cell fusion approach, in which a target cell is fused to a pluripotent stem cell to induce pluripotency within a target nucleus. The cell fusion system is a simple, versatile way to induce reprogramming towards another lineage, not limited to pluripotency. Using this system, the first sign of reprogramming can be detected from within a few hours to one day after fusion, allowing us to monitor the initial events of reprogramming after induction.

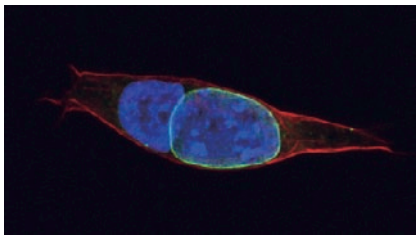


Figure 1. Cellular fusion to study reprogramming: a human lymphoblastoid nucleus can be induced to undergo nuclear reprogramming towards pluripotency upon fusion with mouse ES cells (green). Lamin B1 is endogenously tagged with GFP in ES cells, allowing us to distinguish ES vs lymphoblastoid nucleus during live-imaging (unpublished).

Using this system, we previously found that DNA synthesis is an important event for successful reprogramming (Tsubouchi et al., Cell, 2013). Recent reports indicate that reprogramming may cause genetic instabilities, some of which are thought to arise as DNA replication errors. To investigate the nature of such errors and how they are linked to reprogramming-specific events, we are in the process of setting up a system to isolate and track a single fused cell through live-imaging (Figure 1).

III. Future Perspective

While the fundamental mechanisms that maintain genome integrity have been widely studied using various models, the danger a cell might face when altering their cellular identity (through differentiation, reprogramming etc.) is unknown. Recent studies of cancer genome sequencing repeatedly identified mutations in the factors that govern cellular identities, leading us to hypothesize that cells may experience genome instability when their identity is unstable. Our goal is to uncover the nature of such genetic instability and to gain a comprehensive understanding of the mechanisms that maintain genome integrity.

Publication List:

〔Review article〕

● Tsubouchi, H., Argunhan, B., and Tsubouchi, T. (2016). Shaping meiotic chromosomes with SUMO: a feedback loop controls the assembly of the synaptonemal complex in budding yeast. Microbial Cell *3*, 126-128.

DIVISION OF MORPHOGENESIS

Professor
UENO, Naoto

Associate Professor
KINOSHITA, Noriyuki

Assistant Professor:	*TAKAHASHI, Hiroki*
	SUZUKI, Makoto
Technical Staff:	*TAKAGI, Chiyo*
NIBB Research Fellow:	*YAMAGUCHI, Takeshi**
Postdoctoral Fellow:	*NEGISHI, Takefumi*
	SUZUKI, Miho
	HASHIMOTO, Yutaka
	YAMAGUCHI, Takeshi
SOKENDAI Graduate Student:	*HAYASHI, Kentaro**
	TOMINAGA, Hitoshi
Visiting Scientist:	*HAYASHI, Kentaro*
Technical Assistant:	*YAMAMOTO, Takamasa*
	MURAKAMI, Michiyo
	MIYAGI, Asuka
	YASUE, Naoko
Secretary:	*MIYAKE, Satoko*
	TSUGE, Toyoko

The complex morphogenesis of organisms is achieved by dynamic rearrangements of tissues during embryogenesis, in which change in cellular morphology as well as orchestrated cell movements are involved. For cells to know how they should change their shape and where they should move, information called "cell polarity" is essential. How then is the cell polarity established within cells? Is it intrinsically formed within the cells or triggered by extracellular cues? Furthermore, little is known as to how coordinated and complex cell movements are controlled in time and space. We attempt to understand the mechanisms underlying these events using several model animals, including frogs, fish, mice and ascidians, taking physical parameters such as force in consideration, in addition to conventional molecular and cellular biology.

I. Spatio-temporal regulation of traction force during the collective chemotractic migration

During embryogenesis, the arrangement of multicellular tissue is dramatically changed to establish properly shaped embryos. These movements of group of cells are often highly organized and collective. Investigating the mechanism of collective cell migration is therefore essential for understanding embryogenesis. *Xenopus* leading edge mesoderm (LEM) is one of the suitable models for studying this morphogenetic movement, because of easy micromanipulation of tissue excised from the embryo. During *Xenopus* gastrulation, LEM moves into the blastocoel ahead of the axial mesoderm, which forms the notochord and muscles in the future. In our previous studies, we have shown that LEM generates the driving force of mesodermal migration, and measured the physical value of this force with the explant. Following this, we have started to address how each single LEM cell generates the force for collective migration in the explant. In order to understand this, we have decided to establish Traction Force Microscopy (TFM) for *Xenopus* LEM explant migration. Eventually, we completed the TFM system for this multi-cellular movement with a combination of acrylamide gel and chemoattractants. Our TFM observation revealed that traction force was generated by the cells, which were located at the anterior part of explants. We are investigating how each LEM cell generates traction force in a spatiotemporally regulated manner.

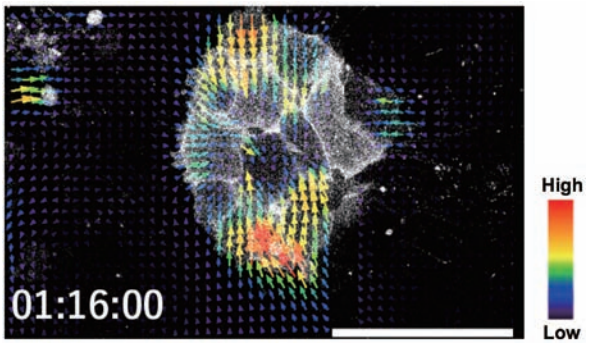


Figure 1. A snapshot of TFM observation of LEM explant migration. LEM explant expressing membrane GFP to visualize the plasma membrane migration toward the left side. The direction of arrow shows that of traction force, size and color indicate the intensity of force.

II. Mechanotransduction in *Xenopus* embryonic cells

During early embryogenesis, a variety of dynamic morphogenetic movements occur, which include convergent extension of the axial mesoderm, epiboly of the ectoderm, and neural tube formation. These movements must generate physical forces at the levels of cells and tissues. However, it is still not fully understood how these forces influence morphogenetic processes. This project is to elucidate the mechanosensing and mechanoresponse of *Xenopus* embryos, and to clarify the roles of mechanotransduction in embryogenesis.

Cells sense mechanical stresses in several ways, for example, with TRP channels, F-actin, cadherins, and/or focal adhesions. Physical stimuli sensed by these molecules are converted to intracellular chemical signals, which in turn induce cellular response. Phosphorylation is thought to be one of the earliest responses to mechanical stresses. Therefore, we attempt to profile protein phosphorylation upon mechanical stimuli and identify the repertoire of target proteins in *Xenopus* embryonic cells. In order to comprehensively analyze levels of protein phosphorylation, we took a phosphoproteomic approach. So far, we have confirmed that a mechanical stress applied to *Xenopus* embryos changes phosphorylation levels of some protein kinases and cytoskeletal regulators. Particularly, some proteins that localize in the cell cortical actin network seem to be the targets of phosphorylation. These results suggest that mechanical stresses may regulate activities of protein kinases and/or protein phosphatases in *Xenopus* embryonic cells, and that the cortical actin network may be involved in this mechanotransduction.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2016. The former title is indicated by an asterisk (*).

III. Mathematical analysis of neural tube formation

During early development of the central nervous system, neuroepithelial cells increase their height by microtubule rearrangement. This cell shape change is called cell elongation, which drives the tubular morphogenesis, called neural tube formation. We previously showed that cell elongation during *Xenopus* neural tube formation depends on orthologs of two microtubule-associated proteins: MID1, which is responsible for Opitz G/BBB syndrome in humans, and its paralog MID2. In this study, to further investigate the role of the cell elongation, we developed a three-dimensional multi-cell-based mechanical model and used it to simulate the process of neural tube formation. Computational simulation of our model suggests that cell elongation drove the rapid folding of the neural plate, and reduced the lumen size of the neural tube. Then we investigated the *in vivo* neural tube by knocking down MID proteins, and found that cell elongation actually reduces the lumen size of the neural tube. These data highlight the function of MID proteins in regulation of the lumen size of the neural tube, and demonstrate the advantage of mathematical analysis for understanding the mechanical basis of morphogenesis.

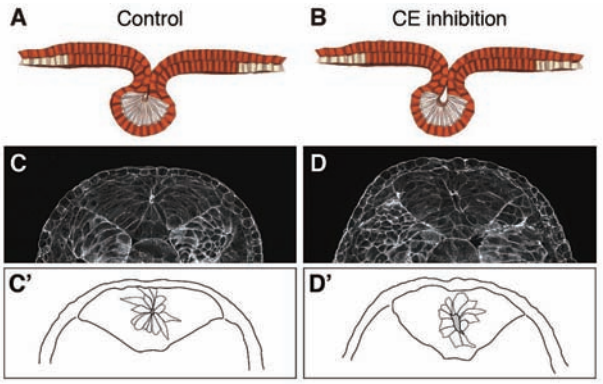


Figure 2. Effects of cell elongation (CE) on modeled epithelial sheets and *in vivo* neural tube.

(A,B) Modeled epithelial sheets at the end of each simulation with cell elongation (A) and without cell elongation (B). (C,D) F-actin staining of control embryo (C) and MID-Mo injected embryo (D). (C') and (D') indicate outlines of neural cells and lumen (gray) of (C) and (D), respectively.

IV. A novel membrane structure orchestrates centrosome positioning and the orientation of cell division axis.

The positioning of the centrosome is critical for the polarity of cilia and the orientation of the mitotic spindle, which are important events during morphogenesis. Previously, in ascidian, *Ciona intestinalis* embryos, we found a unique centrosome-targeting of the membrane structure (membrane invagination) in epidermal cells, which divide stereotypically along the anterior-posterior (A–P) axis. We also reported that membrane invaginations showed the A–P polarity and a tensile force toward the posterior end of cell. By injection of antisense morpholino oligo (MO), we revealed that Dishevelled (Dsh), a core component of Planar Cell Polarity (PCP) pathway was involved in the A–P polarity of membrane invaginations. In the Dsh-depleted embryos, the direction of the invagination as well as mitotic spindle orientation was randomized. In addition, epidermal cilia normally localized in the posterior side of the cell was anteriorized. These results support our hypothesis that the membrane invagination controls the direction of cell division as well as ciliary positioning via the tethering centrosome at the posterior side of the epidermal cell and highlight physiological importance of this unique structure.

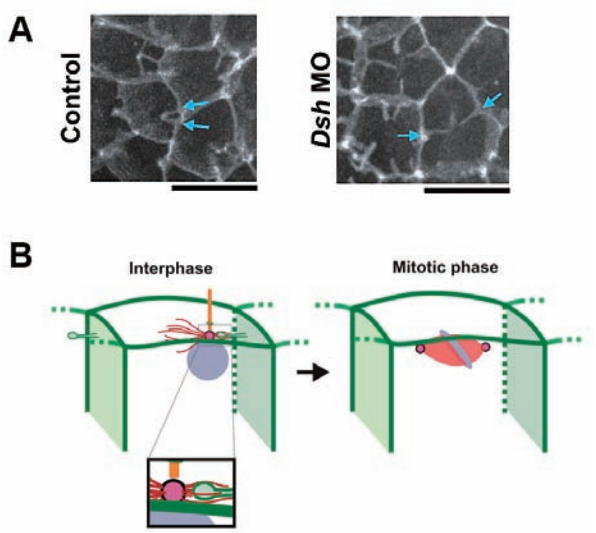


Figure 3. (A) The direction of membrane invagination is disturbed in Dsh-deficient epidermal cells. The plasma membrane was labeled with PH-GFP.

In the epidermal cells of control MO injected *Ciona* embryo, the membrane invaginations show the A-P polarity (Control). In the epidermal cells of *Dsh* MO injected embryo, the direction of invagination is randomized (*Dsh* MO). Blue arrows indicate representative membrane invaginations. Anterior: left. Bar: 10 μm.

(B) Graphical summary of our hypothesis. Anterior is left. Interphase: nuclei (purple), cell membrane (green), centrosome (magenta), microtubule (red) and cilium (orange). The membrane invagination pulls the centrosome/cilium toward the posterior in the interphase, and then mitotic spindle forms aligned along the A-P axis.

V. Notochord and evolution of chordates

A T-box family transcription-factor gene, *Brachyury*, has two expression domains with corresponding functions during animal embryogenesis. The primary domain, associated with the blastopore, is shared by most metazoans, while the secondary domain restricted to the notochord, is specific to chordates.

Therefore, how *Brachyury* acquired its secondary expression domain at the mid-dorsal region of the blastopore and how it is related to the emergence of the notochord is a big question of evolutionary biology. To elucidate how chordate ancestors gained the new expression domains, we used Amphioxus, the most ancestral chordates. We functionally analyzed the Amphioxus *Brachyury* promoter regions by reporter assay, using *Ciona intestinalis* embryos.

As results, we found that some fragments promoted the reporter expression in multiple regions such as blastopore, muscle and notochord, respectively, in ascidians. This result indicates that those Amphioxus enhancers have capacities

of driving *Brachyury* expression in the lateral mesoderm and notochord, in addition to the blastopore of ascidians. Furthermore, we found some other regions drive the expression specifically in the notochord, suggesting that Amphioxus notochord *Brachyury* expression is regulated by the combination of multiple enhancers.

VI. The *Ciona intestinalis* cleavage clock is independent of DNA methylation

DNA methylation at cytosine residues is an important epigenetic modification found in eukaryotes ranging from plants to humans. Invertebrates offer an interesting model for studying evolutionary changes in the targets and the function of DNA methylation. A marine invertebrate chordate *Ciona intestinalis* has a genome-wide mosaic methylation pattern comprising methylated and unmethylated genes. It has been observed that DNA methylation is targeted to the transcribed region of ubiquitously expressed genes, and a constant targeting of "gene body methylation" irrespective of cell types. To reveal the function of gene body methylation in gene transcription, we analyzed newly synthesized RNA from *C. intestinalis* embryos. By using 4sU labeling and sequencing methods, revealing global RNA processing kinetics at nucleotide resolution, we obtained snapshots of active transcription. Significant differences were seen in co-transcriptional splicing efficiency, in connection with methylation status of exons and introns. The splicing efficiency and DNA methylation status were also correlated to nucleosomal positions, suggesting that epigenetic states in the bodies of transcribed genes control the pre-mRNA processing through nucleosomal positioning.

VII. Cnidarian-symbiodinium Symbiosis

Corals are declining globally due to a number of stressors. Such stresses lead to a breakdown of the essential symbiotic relationship between coral and *Symbiodinium*, a process known as coral bleaching. Although the environmental stresses cause this breakdown, the molecular and cellular mechanisms of symbiosis are still unclear. Corals are not very suitable as laboratory systems due to their slow growth, long generation times, and calcareous skeletons. To overcome these limitations, we focused on the small sea anemone *Aiptasia* as a novel experimentally tractable cnidarian model organism. *Aiptasia*, just as reef-building corals, establishes a stable but temperature-sensitive symbiosis with *Symbiodinium*. *Aiptasia* can be repeatedly bleached and repopulated with *Symbiodinium*, grows rapidly, and lacks a calcareous skeleton, allowing microscopic and cell biological analyses.

As the innate immune system recognizes the self and others, it might distinguish *Symbiodinium* as well as pathogens from the host. In fact, the genomic information of *Acropore digitifera,* one of the reef-building corals, revealed that the coral innate immune repertoire is far more complex than that of non-symbiotic cnidarians, *Nematostella* and *hydra*. We thus speculate that the complex innate immune repertoire may reflect adaptation to the symbiotic state. We used chemical inhibitors for the inanate immune signaling, and found that the symbiotic state of coral and *Aiptasia* is influenced by the inhibitors .

Publication List:

〔Original papers〕

- Inoue, Y., Suzuki, M., Watanabe, T., Yasue, N., Takeo, I., Adachi, T., and Ueno, N. (2016). Mechanical roles of apical constriction, cell elongation, and cell migration during neural tube formation in *Xenopus*. Biomech. Model Mechanobiol. *15*, 1733-1746.
- Nagasaka, A., Shinoda, T., Kawaue, T., Suzuki, M., Nagayama, K., Matsumoto, T., Ueno, N., Kawaguchi, A., and Miyata, T. (2016). Differences in the mechanical properties of the developing cerebral cortical proliferative zone between mice and ferrets at both the tissue and single-cell levels. Front. Cell Dev. Biol. *4*, 139.
- Negishi, T., Miyazaki, N., Murata, K., Yasuo, H., and Ueno, N. (2016). Physical association between a novel plasma-membrane structure and centrosome orients cell division. eLife *5*, e16550.
- Sakamaki, K., Ishii, T.M., Sakata, T., Takemoto, K., Takagi, C., Takeuchi, A., Morishita, R., Takahashi, H., Nozawa, A., Shinoda, H., Chiba, K., Sugimoto, H., Saito, A., Tamate, S., Satou, Y., Jung, S.K., Matsuoka, S., Koyamada, K., Sawasaki. T., Nagai, T., and Ueno, N. (2016). Dysregulation of a potassium channel, THIK-1, targeted by caspase-8 accelerates cell shrinkage. Biochim. Biophys. Acta *1863*, 2766-2783.
- Sekiguchi, T., Kuwasako, K., Ogasawara, M., Takahashi, H., Matsubara, S., Osugi, T., Muramatsu, I., Sasayama, Y., Suzuki, N., and Satake, H. (2016). Evidence for conservation of the calcitonin superfamily and activity-regulating mechanisms in the basal chordate *Branchiostoma floridae*: insights into the molecular and functional evolution in chordates. J. Biol. Chem. *291*, 2345-2356.
- Session, A.M., Yamamoto, T.S., Takagi, C., Ueno, N., *et al*. (2016). Genome evolution in the allotetraploid frog *Xenopus laevis*. Nature *538*, 336–343.
- Suzuki, M., Takagi, C., Miura, S., Sakane, Y., Suzuki, M., Sakuma, T., Sakamoto, N., Endo, T., Kamei, Y., Sato, Y., Kimura, H., Yamamoto, T., Ueno, N., and Suzuki, K.T. (2016). *In vivo* tracking of histone H3 lysine 9 acetylation in *Xenopus laevis* during tail regeneration. Genes Cells *21*, 358-369.
- Suzuki, M.M., Mori, T., and Satoh, N. (2016). The *Ciona intestinalis* cleavage clock is independent of DNA methylation. Genomics *108*, 168-176.

DIVISION OF MOLECULAR AND DEVELOPMENTAL BIOLOGY

Professor
TAKADA, Shinji

Assistant Professor:	*YABE, Taijiro*
	MII, Yusuke
Technical Staff:	*UTSUMI, Hideko*
NIBB Research Fellow:	*OKADA, Kazunori*
Postdoctoral Fellow:	*TAKADA, Ritsuko*
	FUJIMORI, Sayumi
SOKENDAI Graduate Student:	*SHINOZUKA, Takuma*
	TSUCHIYA, Yoshihiro
Visiting Scientist:	*CHEN, Qiuhong*
Technical Assistant:	*TAKASHIRO, Kayoko*
	ITO, Yukiko
Secretary:	*UKAI, Sakie*
	NOBATA, Ryuko

The morphology of the body and tissues is established in spatio-temporarily regulated manners. A number of genes involved in morphogenesis have been identified, but it is still uncertain how the spatial and temporal information are established and converted to morphology during embryogenesis. Therefore, we are challenging to understand the mechanism by which the spatial information is established and that by which the temporal, or periodical, information is converted into morphology by several different approaches.

In the development of many tissues, secreted signal molecules are important for the formation of spatial information. These molecules are secreted from producing cells and transported to surrounding cells, resulting in the formation of concentration gradients. Given that their concentration decreases according to the distance from the source, the gradient of the signals defines relative positions of receiving cells in developing tissues. Many genetic studies revealed that a limited number of secreted signal proteins, including Wnt, BMP, and Hedgehog, function in the morphogenesis of tissues and embryos. In spite of the accumulation of genetic evidence, however, the molecular mechanism that regulates their spread in particular developing tissues remains to be elucidated. To this end, we started to visualize signal proteins and monitor their movement in tissues. In addition, we are examining structural and biochemical characteristics of these molecules, which appear to affect how they spread.

The segmental sub-regions of several specific tissues, including the paraxial mesoderm (or somites) and the pharyngeal pouches, by contrast, appear not to be simply directed by the gradient of the signal molecules, but by a unique mechanism proceeding periodically. For instance, somites are sequentially generated in an anterior-to-posterior order by converting the temporal periodicity created by a molecular clock into periodical structures. The molecular mechanism underlying this conversion and morphological segmentation, however, is not yet fully understood. Thus, another goal of our current studies is to reveal the molecular mechanism of this other and unique mode of patterning that underlies the periodical and sequential sub-division in the development of somites and pharyngeal arches.

I. Spatial regulation of secreted Wnt proteins in vertebrate development

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis. In a classical view, it has been proposed that secreted signal proteins, like Wnt proteins, spread and function over a distance of several cells in many aspects of morphogenesis. In contrast, accumulating evidence also implies that Wnt proteins appear to transmit their signals locally in some particular cases. Therefore, their secretion and transport might be differently controlled depending on situation. Thus, for understanding the spatial regulation of tissue morphogenesis, the molecular mechanism underlying the spreading of Wnt proteins should be revealed.

For better understanding the spreading of Wnt proteins, we started to visualize Wnt proteins in the extracellular space by several different approaches. Our preliminary study indicated that Wnt proteins are not simply diffused during embryogenesis of the mouse. We precisely examined regulatory mechanisms and biological significance of Wnt protein distribution in mouse embryos. These analyses revealed a novel view of spatial regulation of Wnt signaling.

Extracellular molecules are known to regulate special distribution of Wnt proteins. Once Wnt proteins are secreted from the producing cell, their spread is regulated through interaction with these molecules. The heparan sulfate proteoglycan (HSPG), which is composed of a core protein with several chains of HS glycosaminoglycans, is a major component involved in this interaction. In collaboration with Prof. Taira at the University of Tokyo, we found that HSPGs with different sugar chain modifications form distinct extracellular structures, which are called heparan sulfate nanostructures (HSNSs), in the early *Xenopus* embryo.

II. Heterogeneity of secreted Wnt proteins secreted from culture cells

Although the structure of Wnt protein has already been revealed by X-ray crystallography, its higher order structure in extracellular space has not yet been fully understood. One of the proposed forms of Wnt proteins in extracellular space is binding to lipoprotein particles. On the other hand, recent studies indicated that Wnt proteins are secreted on another lipid-based carrier, called the exosome, which is an MVB (multivesicular body)-derived membrane vesicle (Figure 1). However, it remains unclear whether Wnt proteins are secreted only in these two forms, or also in some other forms from the same cells, or if different forms of Wnt proteins are secreted in a cell type specific manner.

To address this question, we systematically examined characteristics of Wnt proteins secreted from polarized MDCK (Madin-Darby Canine Kidney) cells. Although some Wnt3a proteins from mouse L cells were secreted together with lipoprotein particles, most of the Wnt3a proteins from either apical or basolateral side of MDCK cells did not. In contrast, secretion of a small amount of Wnt3a proteins via exosomes was detected. We found that different populations

of exosomes or exosome-like vesicles were released from MDCK cells depending on the cell polarity. Wnt3a associated with these vesicles was detectable in culture media collected from both apical and basolateral sides of the cells. Basolaterally secreted Wnt3a existed with typical exosomes. In contrast, most of apically secreted Wnt3a, as well as Wnt11, existed with non typical exosomes, whose density was higher than that of typical exosomes. The lipidation of Wnt3a was required for its basolateral secretion in exosomes but was dispensable for the apical secretion. These results indicated that epithelial cells release Wnt via distinct populations of vesicles differing in secretion polarity and lipidation dependency.

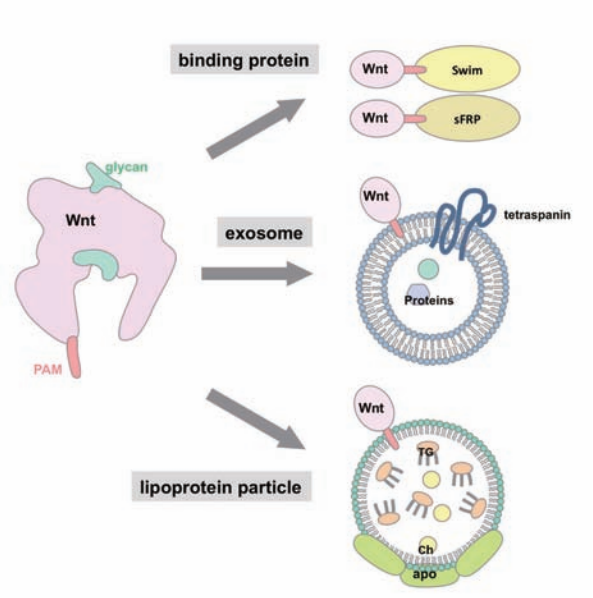


Figure 1. Heterogeneity of Wnt transport system. Secreted Wnt proteins are transported by several different ways, including binding with carrier proteins, associating with exosomes, or loading on lipoprotein particles.

III. Molecular mechanism of somite development

Somites are segmental epithelial blocks located symmetrically on either side of the neural tube. Somites are periodically generated in an anterior to posterior manner from their precursor, the presomitic mesoderm (PSM), which is located posterior to newly formed somites. This periodic generation is achieved by a complex and dynamic mechanism in the PSM. First, the molecular clock, the so-called segmentation clock, creates oscillatory expression of particular genes, *hairy* and some *notch*-related genes, in the posterior PSM. Because the phase of oscillation is gradually shifted along the posterior-to-anterior axis, a wave of the oscillation appears to move in a posterior-to-anterior fashion. This oscillatory gene expression subsequently resulted in periodical generation of morphologically segmented somites.

The spatial pattern of somites is defined by positioning of intersomitic boundaries and rostro-caudal patterning within a somite. This spatial pattern is established in the anterior PSM by converting the oscillatory gene expression into spatially restricted patterns of gene expression. Several intra-cellular molecules, as well as FGF and Wnt signaling, are involved in this conversion. In the mouse, the prospective segmentation boundary is periodically established in the anterior PSM at the rostral border of the *Mesp2* expression domain. Mesp2, one of the key regulators in this conversion, is initially expressed at the most anterior region of the Tbx6 protein domain. This expression is not anteriorly extended beyond the anterior border of the Tbx6 protein domain because *Mesp2* expression requires Tbx6 proteins. Thus, the anterior border formation of the Tbx6 protein domain is a more fundamental process in the positioning of the segmentation boundary.

Importantly, this border is not consistent with the anterior border of *Tbx6* mRNA, rather it is regulated by a proteasome-mediated mechanism. Although the molecules directly executing this proteolysis are still unclear, *Mesp2*, as well as *Ripply1* and *Ripply2*, have shown to be required for the down-regulation of Tbx6 proteins by analysis of mouse embryos defective in these genes. Since expressions of *Ripply1* and *2* are eliminated in *Mesp2* deficient mouse embryos, we previously proposed the following model; *Mesp2,* whose expression is activated in the most anterior part of the Tbx6 domain, causes retreat of the Tbx6 protein domain through activation of *Ripply1* and *2* expression, and

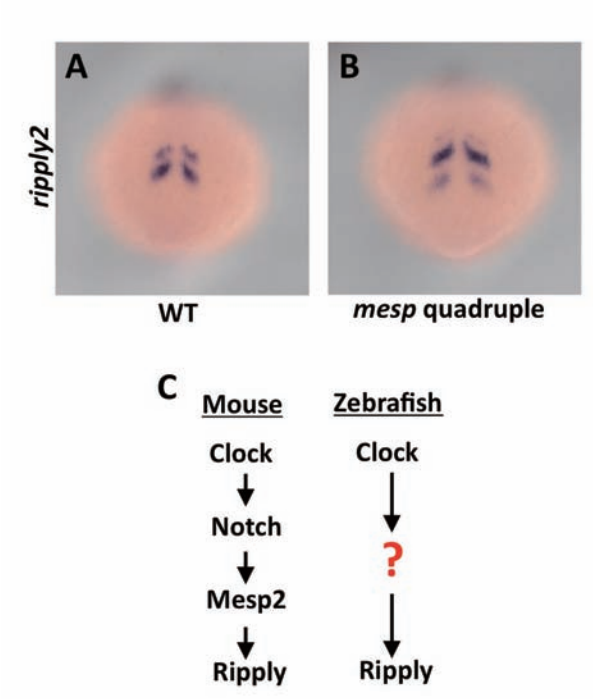


Figure 2. (A, B) Expression of *ripply2* in WT embryo (A) and *mesp* quadruple mutant embryo (B). In contrast to mice, the function of mesp is not required for the *ripply* expression in zebrafish. (C) Scheme of the regulation of *ripply* expression by the segmentation clock. In the mouse, temporal information generated by segmentation clock is converted to the expression pattern of *ripply* by the functions of Notch and Mesp2. However, this molecular machinery is not conserved between mouse and zebrafish and molecular mechanism regulating *ripply* expression still remains to be elucidated.

the retreated Tbx6 subsequently defines the next segmentation border and *Mesp2* expression. Recently, we showed that Ripply is a direct regulator of the Tbx6 protein level for the establishment of intersomitic boundaries. However, it is still to be elucidated whether *Mesp* is actually required for the formation of the boundaries in zebrafish.

To answer this question, we generated zebrafish mutants lacking all 4 zebrafish *mesp* genes by a genome-editing approach. In contrast to the case for the mouse *Mesp2* mutant, the positions of somite boundaries were normal in the zebrafish *mesp* quadruple mutant embryos. On the other hand, each somite was caudalized similarly to the mouse *Mesp2* mutant. These results clarify the conserved and species-specific roles of Mesp in the connection of the molecular clock to somite morphogenesis (Figure 2).

IV. Molecular mechanism of pharyngeal pouches

In addition to somites, metameric structures are observed in the pharyngeal region of vertebrates. Typical examples of such structures are skeletal elements of jaws, gills and cranial nerve projections. This metamerism is brought about by the segmental development of the pharyngeal pouches, which are generated by outpocketing of the pharyngeal endoderm. However, the molecular mechanisms underlying the segmentation of the pharyngeal pouches and the morphogenesis of the pharyngeal pouches still remained to be elucidated.

To understand these mechanisms, we examined the development of pharyngeal pouches in medaka embryos in collaboration with Prof. Wada at Tsukuba University. We found that the expression of *pax1* in the endoderm prefigured the location of the next pouch before the cells bud from the epithelium. Embryos deficient for *pax1* did not form the pharyngeal pouches posterior to the second arch. Precise analysis of gene expression suggests that *pax1* has a critical role in generating a primary pattern for segmentation in the pharyngeal endoderm (Figure 3).

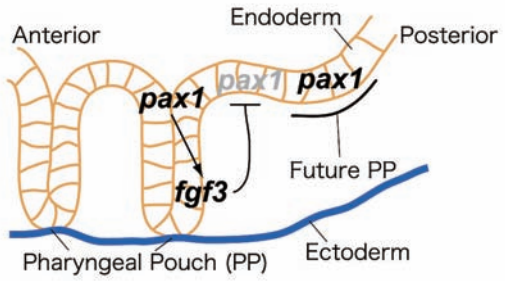


Figure 3. Model of molecular mechanism of the segmentation of pharyngeal pouches in the medaka embryo. Our results suggest that interaction between pax1 and fgf3 is a key process for generation of the segmental pattern of pharyngeal pouches.

Publication List:

〔Original papers〕

- Chen, Q., Takada, R., Noda, C., Kobayashi, S., and Takada, S. (2016). Different populations of Wnt-containing vesicles are individually released from polarized epithelial cells. Sci. Rep. *6*, 35562.
- Kawamura, A., Ovara, H, Ooka, Y., Kinoshita, H., Hoshikawa, M., Nakajo, K., Yokota, D., Fujino, Y., Higashijima, S.I., Takada, S., and Yamasu, K. (2016). Posterior-anterior gradient of zebrafish hes6 expression in the presomitic mesoderm is established by the combinatorial functions of the downstream enhancer and 3'UTR. Dev. Biol. *409*, 543-554.
- Ohta, Y., Kamagata, T., Mukai, A., Takada, S., Nagai, T., and Horikawa, K. (2016). Nontrivial effect of the color-exchange of a donor/acceptor pair in the engineering of Förster resonance energy transfer (FRET)-based indicators. ACS Chem. Biol. *11*, 1816-1822.
- Okada, K.*, Inohaya, K., Mise, T., Kudo, A., Takada, S.*, and Wada, H.* (2016). Reiterative expression of pax1 directs pharyngeal pouch segmentation in medaka. Development *143*, 1800-1810. (*: Co-corresponding authors)
- Takemoto, T., Abe, T., Kiyonari, H., Nakao, K., Furuta, Y., Suzuki, H., Takada, S., Fujimori, T., and Kondoh, H. (2016). R26-WntVis reporter mice showing graded response to Wnt signal levels. Genes Cells *21*, 661-669.
- Yabe, T., Hoshijima, K., Yamamoto, T., and Takada, S. (2016). *Mesp* quadruple zebrafish mutant reveals different roles of *mesp* genes in somite segmentation between mouse and zebrafish. Development *143*, 2842-2852.

〔Review articles〕

- Kondoh, H., Takada, S., and Takemoto, T. (2016). Axial level-dependent molecular and cellular mechanisms underlying the genesis of the embryonic neural plate. Dev. Growth Differ. *58*, 427-436.
- Yabe, T., and Takada, S. (2016). Molecular mechanism for cyclic generation of somites: Lessons from mice and zebrafish. Dev. Growth Differ. *58*, 31-42.

Division of Embyology

Professor
FUJIMORI, Toshihiko

Assistant Professor:	*TOYOOKA, Yayoi*
	KOYAMA, Hiroshi
Technical Staff:	*OKA, Sanae*
NIBB Research Fellow:	*SHI, Dongbo*
Postdoctoral Fellow:	*DAY, F. Timothy*
	NAKANOH, Shota
SOKENDAI Graduate Student:	*KAMEMIZU, Chizuru*
	ITO, Tomoaki
	USAMI, Fumiko
	OTAKE, Norihito
Technical Assistant:	*HIGUCHI, Yoko*
	NAKAGAWA, Mami
Secretary:	*KATO, Azusa*

The aim of our research is to understand the events underlying early mammalian development during the period from pre-implantation to establishment of the body axes.

Early events during embryogenesis in mammals are relatively less understood, as compared to other animals. This is mainly due to difficulties in approaching developing embryos in the oviducts and the uterus of the mother. Another characteristic of mammalian embryos is their highly regulative potential. The pattern of cell division and allocation of cells within an embryo during the early stages vary between embryos. The timing of the earliest specification events that control the future body axes is still under investigation. Functional proteins or other cellular components have not been found that localize asymmetrically in the fertilized egg. We want to provide basic and fundamental information about the specification of embryonic axes, differentiation of cell lineages, behaviors of cells, and the regulation of body shape in early mammalian development. We are also interested in the relationship between embryos and their environment, the oviducts and the uterus.

I. Live observation of early mouse development

To understand the mechanisms underlying embryonic development and morphogenesis, it has become common to observe the behavior of cells and gene expression in living embryos in a variety of animal species. Progress in GFP technologies, genetic engineering and embryonic manipulation concomitantly with the progress in optics and microscope technologies, including development of highly sensitive photo-detectors, has allowed us to observe developing embryos live, even in mammals. We have established a series of transgenic mouse lines for live imaging, which is part of a collaborative project taking place in the Laboratory for Animal Resources and Genetic Engineering, Riken CLST. These mouse lines have been used to visualize nuclei, cell shapes, the cytoskeleton and other organelles to observe cell behaviors in living mouse embryos in many laboratories over the world. We also established mouse lines to monitor the cell cycle.We have also been establishing several reporter mouse lines in the lab to study gene expression patterns during the peri-implantation stage of mouse development. In these mice, cDNA encoding fluorescent proteins are connected to the mouse genomic DNA sequences of the enhancer/promoter region of important genes encoding factors regulating cell differentiation in these stages. Combining these two types of reporter transgenic mouse lines, we have been analyzing behaviors of cells comparing gene expression properties at the single cell level.

We have been trying to observe and reveal aspects of cell shape, morphogenesis, cell lineage, gene expression and cell differentiation in developing embryos and in other tissues by combining these techniques. To monitor cell movement and the status of the trophectoderm (TE) specification pathway in living embryos, we established Cdx2-GFP reporter mice allowing us to visualize the expression of Caudal-type transcriptional factor (Cdx2), a key regulator of the initiation of TE differentiation. Cells localized in an outer position initiated the expression of Cdx2. Subsequently, cells that changed their position from an outer to an inner position down-regulated Cdx2 expression and contributed to the pluripotent inner cell mass (ICM). Our results indicate that cells expressing even high levels of Cdx2 can internalize, deactivate the TE-specification molecular pathway and be integrated into the pluripotent cell population. This suggests that cells expressing Cdx2 exhibit plasticity of specification to TE and ICM lineages through positional changes.

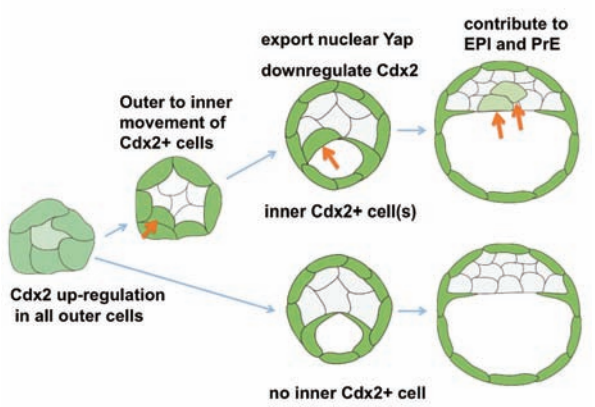


Figure 1. Cdx2 expressing cells in preimplantation mouse embryo exhibit plasticity of specification. Outer positioned cells up-regulate Cdx2 expression, but some internalized cells down-regulate its expression and contribute to both pluripotent epiblast (Epi) and extra-embryonic primitive endoderm (PrE).

II. Histological observation of mouse embryos developing in the uterus

One of the characteristics of mammalian development is that embryos develop in the uterus after implantation. Direct interaction between embryos and the cells of the uterus is important. In studies on developmental biology of mammalian embryos, embryos are usually removed from the uterus, and isolated embryos are analyzed. We have been analyzing early embryonic development of mouse comparing the changes in the uterine epithelium. To observe the morphogenesis of embryos within the uterus, serial sections of implanted uteruses were made, stained with hematoxylin and eosin, and images of the embryos within the uteruses were captured to make high resolution three-dimensional

re-constructions. Figure 2 shows an example of a section. We are preparing samples obtained from various developmental stages. Precise positions of embryonic implantation and changes in the uterine epithelium correlating with embryonic development will be examined using these images. To obtain three dimensional images of embryos within the uterus, we have been developing a system to automatically extract regions of the uterus where embryos localize by utilizing image analysis after images of serial sections are captured using a slide scanner.

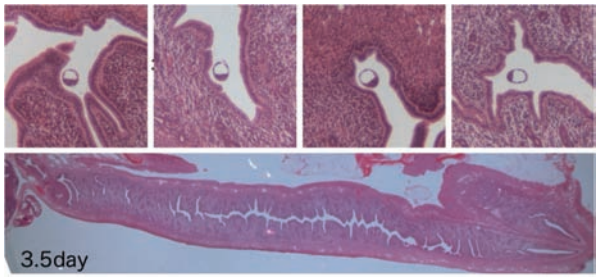


Figure 2. A pregnant uterus 3.5 days after fertilization. Developing embryos and the shape of uterus epithelium can be observed.

III. Polarity formation in the mouse oviduct epithelium

The oviduct (Fallopian tube) is the organ connecting the periovarian space and the uterine horn. The ova released from the ovary are transported through the oviduct, where the ova are fertilized by spermatozoon entering from the uterus. We are focusing on the region of the oviduct close to the opening to the ovary.

The epithelium of the mouse oviduct consists of multi-ciliated cells and secretory cells. Ovulated oocytes are transported in oviducts by unidirectional motility of multi-cilia and the resultant secretory fluid flow from ovary to uterus. This cellular orientation in the oviduct is one example of planar cell polarity (PCP). PCP is seen in many animals and tissues, where cells sense global axes of the tissue to which they belong and orient themselves to fulfill specialized functions. Many evolutionally conserved genes are required for the formation of PCP, and several of those encode proteins that are localized in polarized manners within cells. We found that Celsr1, a PCP core member, was strongly concentrated only at the specific domains of cell boundaries which are perpendicular to the ovary-uterus axis and that this polarized localization appeared to precede the directional movement of cilia.

In *Celsr1*-deficient mutant oviducts, cilia were generated and those within each cell appeared to beat as in the wild type oviduct. However, the beating direction was not coordinated along the ovary-uterus axis throughout the epithelia; some cells were oriented orthogonally to the axis, while some others exhibited reverse polarity. In addition to the ciliary polarity phenotype, cellular shape polarity was also impaired in the *Celsr1*-deficient mice. We found a new feature of cellular polarity in the wild type oviduct, e.g. that the apical surface of epithelial cells was elongated along the ovary-uterus axis. In *Celsr1*-deficient mice, epithelial cells showed less elongation and randomized orientation.

Furthermore, we also found that the three-dimensional architecture of oviductal epithelia was dramatically disrupted. In adult wild-type mice, the epithelial cell sheet is bent multiple times, which forms around twenty longitudinal folds parallel to the organ axis. However, in the mutant oviducts, epithelial folds no longer run parallel to the organ axis. The direction of the folds was randomized, and ectopic branches of the folds were observed. This suggests that Celsr1 is important for the formation of multi-scale polarities in the mouse oviduct ranging from the cellular to the tissue scale. To investigate the mechanisms of the epithelial fold pattern formation, we utilized mathematical modeling and simulations. By considering mechanical properties of the epithelial sheets we reproduced the longitudinally aligned folds and the branched folds which are observed in wild-type and the *Celsr1* mutant mice, respectively (Figure 3). Experimental measurements of mechanical tensions in the epithelial sheet were consistent with the tensions predicted from the simulations. Our experimental and mathematical analyses also successfully linked the epithelial tensions to cellular shapes. We are also focusing on some other PCP regulators, and are trying to understand how oviduct epithelial cells establish and maintain their polarity.

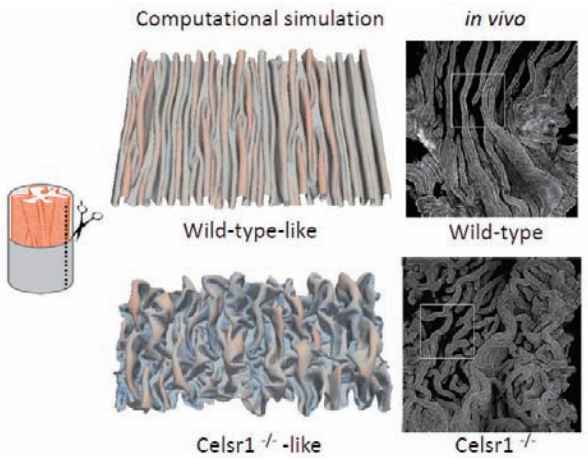


Figure 3. Epithelial fold patterns in oviduct and the reproduction of the patterns by computational simulations.

IV. Analysis of mechanical properties of cells and tissues during embryonic development

Mechanics is one of the essential components of biological processes, including cell shape transformation and tissue morphogenesis etc. To understand how mechanical forces contribute to various patterns, measuring cellular and tissue mechanical states are necessary. We developed statistic techniques to infer the mechanical states by using fluorescent microscopic images during morphogenesis (Figure 4). By employing this method, we inferred mechanical forces in multi-cellular systems including MDCK cultured cells, and early embryogenesis in *C. elegans* and mice. Further computational simulations based on the inferred mechanical information reproduced morphological features of the multi-cellular systems. Thus, the mechanical information will be useful to investigate physical mechanisms of early embry-

onic development and morphogenesis during organogenesis in late stages of development.

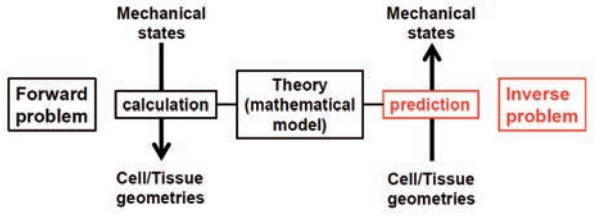


Figure 4. Statistical inference of cellular/tissue mechanical states.

Publication List:

〔Original papers〕

- Koyama, H., Shi, D., Suzuki, M., Ueno, N., Uemura, T., and Fujimori, T. (2016). Mechanical regulation of three-dimensional epithelial fold pattern formation in the mouse oviduct. Biophys. J. *111*, 650-665.
- Shi, D., Usami, F., Komatsu, K., Oka, S., Abe, T., Uemura, T., and Fujimori, T. (2016). Dynamics of planar cell polarity protein Vangl2 in the mouse oviduct epithelium. Mech. Dev. *141*, 78-89.
- Takemoto, T., Abe, T., Kiyonari, H., Nakao, K., Furuta, Y., Suzuki, H., Takada, S., Fujimori, T., and Kondoh, H. (2016). R26-WntVis reporter mice showing graded response to Wnt signal levels. Genes Cells *21*, 661-669.
- Toyooka, Y., Oka, S., and Fujimori, T. (2016). Early preimplantation cells expressing Cdx2 exhibit plasticity of specification to TE and ICM lineages through positional changes. Dev. Biol. *411*, 50-60.

〔Review article〕

- Shi, D., Arata, M., Usui, T., Fujimori, T., and Uemura, T. (2016). Seven-pass transmembrane cadherin CELSRs, Fat4 and Dchs1 cadherins: From planar cell polatiry to three-dimensional organ architecture. In The Cadherin Superfamily, S. Suzuki, and S. Hirano, eds., pp. 251-275.

DIVISION OF GERM CELL BIOLOGY

Professor
YOSHIDA, Shosei

Assistant Professor:	*KITADATE, Yu*
	NAKAGAWA, Toshinori
Technical Staff:	*MIZUGUCHI, Hiroko*
NIBB Research Fellow:	*HIRA, Seiji*
JSPS Researcher:	*NAKAMURA, Yoshiaki*
Postdoctoral Fellow:	*IKAMI, Kanako*
	TOKUE, Moe
SOKENDAI Graduate Student:	*NONAMI, Yuta*
	ISHIZAKA, Miho
	HIRANO, Kodai
Visiting Graduate Student:	*SATO, Toshiyuki*
Technical Assistant:	*KON, Yayoi*
	MARUYAMA, Ayumi
	NISHIMURA, Keiko
Secretary:	*KUBOKI, Yuko*

Overview of our research

Production of numerous sperm for a long period in testis is fundamental for continuity of life across generations. This process of spermatogenesis is supported by the robust function of "stem cells", which both maintain the undifferentiated cell pool, while generating differentiation-destined cells in a well balanced manner. The Division of Germ Cell Biology aims to fully understand the mouse sperm stem cell system under the context of *in vivo* testicular tissue. Our particular interests have been laid on the "undifferentiated spermatogonia", which are responsible for the stem cell functions. Our study has revealed several key properties of this interesting population.

First, we found that this stem cell system includes a functional hierarchy. It is comprised of an "actual" stem cell compartment that is prone to self-renew, and a differentiation-primed, "potential" stem cell compartment. Regarding the "actual" stem cells, we have been investigating their cellular identity, their *in vivo* behavior at a single-cell resolution, and the underlying mathematical principles. This lead to the discovery of neutral competition between the stem cells. We are currently investigating the molecular mechanism underlying their control of self-renewal and differentiation, and their connection to tissue environment. "Potential stem cells" are also of our enthusiastic interest: In undisturbed, steady-state spermatogenesis, they largely contribute as transient amplifying cells and differentiate rather than self-renew. However, when tissues are insulted or testicular cells are transplanted to a host testes, their probability of self-renewal jumps up and they effectively replenish the lost "actual" stem cells. Such a flexible, and probabilistic, feature of stem cell dynamics has been found paradigmatic for many other stem cell-supported tissues.

Key references for these studies that are currently public include Nakagawa et al., Dev. Cell 2007; Yoshida et al., Science 2007; Nakagawa et al., Science 2010; Klein et al., Cell Stem Cell 2010; and Hara et al., Cell Stem Cell 2014, and Ikami et al., Development 2015.

I. The identity of spermatogenic stem cells and their dynamics

Morphologically, the population of A_{undiff} includes singly isolated cells (A_s), or syncytia consisting mainly of 2 (A_{pr}), 4 (A_{al-4}), 8 (A_{al-8}), or 16 (A_{al-16}) cells. The formation of syncytia is due to incomplete cell division, a germline-specific cell division process where cytokinesis does not complete and the cytoplasmic connection between daughter cells persists via intercellular bridges. The prevailing stem cell theory proposed in 1971 states that stem cell activity is restricted to the population of A_s cells, while interconnected A_{pr} and A_{al} syncytia are irreversibly committed to differentiation and no longer contribute to the stem cell pool (Huckins, 1971; Oakberg, 1971), known as the "A_s model".

Figure 1 represents our latest proposed model for the functional structure of the spermatogenic stem cell system, which indeed proposes an alternative for the "A_s model". This is the simplest interpretation of the results of our functional analyses of GFRα1$^+$ spermatogonia, which act as the "actual" stem cells. These include intravital live-imaging experiments, clonal fate analysis of pulse-labeled cells, and biophysical modeling analysis of the results.

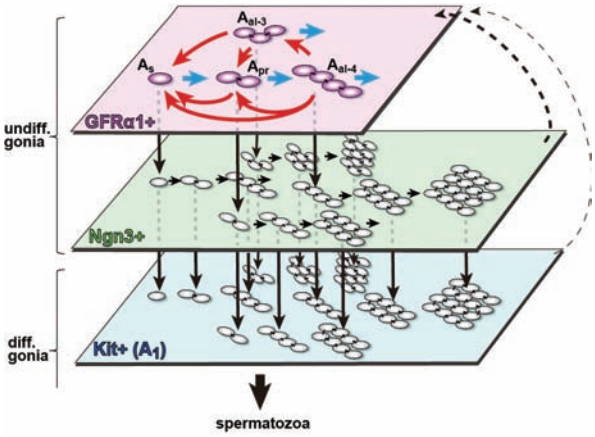


Figure 1. A proposed stem cell dynamics. On the top of the differentiation hierarchy, GFRα1$^+$ spermatogonia comprise a single stem cell pool, in which cells continually and reversibly interconvert between states of A_s, A_{pr} and A_{al} spermatogonia through incomplete cell division (blue arrows) and syncytial fragmentation (red arrows), while giving rise to NGN3$^+$ cells. After leaving the GFRα1$^+$ compartment, differentiation-destined cells follow a series of transitions (GFRα1$^+$→NGN3$^+$→KIT$^+$; downward black arrows) that accompany the extension of syncytial length (rightward black arrows). Ngn3$^+$ and, to a lesser extent, KIT+ cells retain the capacity to revert back into the GFRα1+ compartment in a context-dependent fashion (broken arrows). (Reprinted from Hara et al., Cell Stem Cell 2014.)

As crystalized in this model, our results suggest that the GFRα1$^+$ sub-population of A_{undff} spermatogonia, which include both A_s cells and syncytia (A_{pr} and A_{al}) comprises a single stem cell pool, in which cells continually interconvert between these morphologically heterogeneous states through stochastic incomplete division and fragmentation of syncytia. The incomplete division and syncytial fragmentation causes the expansion of this population, while the excess cells over a particular "quota" would overflow to become the NGN3$^+$ state of A_{undff}, which then further differentiate into KIT$^+$

"differentiating" spermatogonia that are largely devoid of self-renewing potential. Currently, we are investigating the mechanism that determines the quota or tissue capacity of GFRα1⁺ cells, as well as the detailed nature of the internal heterogeneity of the population of GFRα1⁺ cells.

2. Mechanisms underlying the balanced differentiation and self-renewal of stem cells in an "open" stem cell niche

In general, stem cells support tissue homeostasis through continual production of differentiating progeny from a pool of undifferentiated cells. This is traditionally thought to be dependent on a couple of paradigmatic mechanisms: 1) "asymmetric cell division", which always gives rise to one self-renewing cell and one differentiating cell; 2) control by "an anatomically defined niche", inside of which stem cells remain undifferentiated, but they differentiate outside (Fuller and Spradling, 2007; Morrison and Spradling, 2008). However, the mouse spermatogenic stem cell system does not show a defined niche environment and does not rely on asymmetric division; their fates are stochastic (Hara et al., 2014; Klein et al., 2010; Klein and Simons, 2011).

In 2017, we challenged a fundamental question: How sperm stem cells follow different fates (to differentiate or to self-renew) in response to homogeneously distributed extracellular signals, in a facultative (or open) niche environment? The result is published in Tokue et al., Stem Cell Reports (2017).

2-1. Wnt/β-catenin signaling promotes differentiation of GFRα1⁺ to NGN3⁺ cells

To unveil the signal that promotes the GFRα1⁺ to NGN3⁺ transition, we first compared the gene expression profiles between GFRα1⁺ and NGN3⁺ cells by cDNA microarray. From the microarray data, we focused on Wnt signaling. In cultured spermatogonia (viz. GS cell), Wnt/β-catenin signaling induced *Ngn3* expression (Figure 2A). Moreover, mice carrying a gain-of-function mutation of *β-catenin* showed spermatogenesis defects, in which the number of GFRα1⁺ cells was reduced (Figure 2B), whereas no significant reduction in retinoic acid receptor gamma (RARγ)⁺ cells (largely corresponding to NGN3⁺ cells) was observed. Consequently, the RARγ⁺ cell-to-GFRα1⁺ cell ratio increased, consistent with the idea that GFRα1⁺ to NGN3⁺ differentiation increased.

2-2. a cell-autonomous Wnt inhibitor, is expressed in a subset of GFRα1⁺ cells

We found that *Wnt6* is expressed in Sertoli cells, a supporting somatic cell type, in a spatially ubiquitous manner, suggesting that WNT6 is participating in the differentiation-promotion in seminiferous tubules, and that GFRα1⁺ cells are uniformly exposed to WNT6 signals. These raised a next question: How do stem cells persist without exhaustion if they are equally bathed with Wnt ligand(s)?

To address this issue, we hypothesized that a Wnt inhibitor(s) may confer resistance to Wnt/β-catenin signaling in some fraction of GFRα1⁺ cells. In our microarray data, we found *Shisa6* tobe an interesting candidate, because *Shisa6* was highly enriched in the GFRα1⁺ fraction and some of

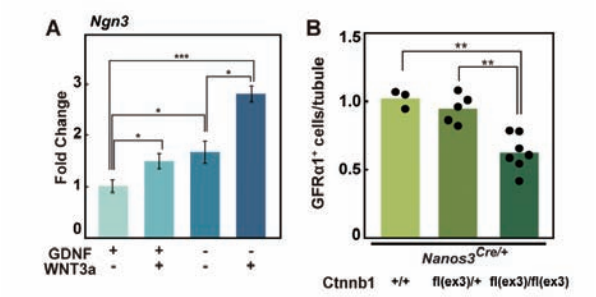


Figure 2. Wnt/β-catenin signal promotes the differentiation of GFRα1⁺ spermatogonia *in vitro* and *in vivo*. (A) Expression of *Ngn3* mRNA in GS cells in the presence or absence of GDNF or WNT3a. GS cells were stimulated by the indicated conditions and analyzed by RT-qPCR for the *Ngn3* mRNA level. (B) Average numbers of GFRα1⁺ cells per tubule, observed in mice with indicated genotypes. Number of GFRα1⁺ cells was reduced in *Nanos3*$^{Cre/+}$;*Ctnnb1* $^{fl(ex3)/fl(ex3)}$ *mice*, in which β-catenin was activated in a germ cell specific manner. Modified from Tokue et al., Stem Cell Reports (2017),

the other *Shisa* family members have been reported as Wnt inhibitors. Experiments using *Xenopus laevis* embryos and luciferase assays in HEK293T cells showed that SHISA6 is a novel Wnt inhibitor that acts autonomously (Figure 3A). Moreover, *Shisa6* expression was restricted to about 30% of the GFRα1⁺ population *in vivo* (Figure 3B).

2-3. SHSIA6⁺ spermatogonia show stem cell characters and contribute to maintenance of GFRα1⁺ cells

We found that, although *Shisa6* KO or stabilized *β-catenin* heterozygous mutants show no apparent phenotype in the pool of GFRα1⁺ cells, synthetic heterozygotes for these alleles showed a reduced GFRα1⁺ cell pool and spermatogenesis defects. Thus, SHISA6 plays a role in the maintenance of the stem cell pool by suppressing Wnt/β-catenin signaling in a cell-autonomous manner (Figure 4A).

We then addressed the behavior of SHISA6⁺ cells conjectured from that of *T* (*Brachyury*)⁺ cells, which were found to largely overlap with SHISA6⁺ cells. A pulse-label and chase experiment showed that T⁺ cells have the ability to continu-

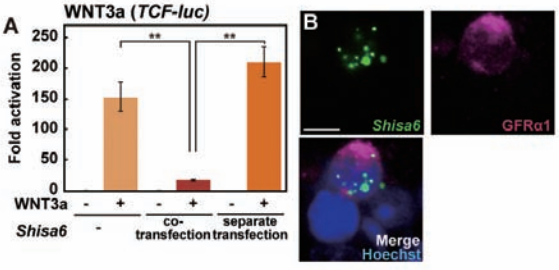


Figure 3. Function and expression of SHISA6 (A) Cell-autonomous inhibition of Wnt/β-catenin signaling by SHISA6. HEK293T cells were transfected with a Wnt reporter luciferase construct and a *Shisa6* expression plasmid either simultaneously (co-transfection), or separately (transfected cells were mixed afterword), followed by stimulation of Wnt/β-catenin signal. The Wnt-dependent Luciferase activity was suppressed only when Shisa6 was co-transfected. (B) Representative image of dissociated testicular cells of adult mice doubly stained for *Shisa6* by FISH and for GFR (B) Representative image. Note the overlapped expression of *Shisa6* and GFRsa is modified from Tokue et al., Stem Cell Reports (2017),

ally produce progeny differentiating to sperm for at least 6 months. Together, these results suggest that T+ (and probably SHISA6+) cells have stem cell-related characteristics (Figure 4B).

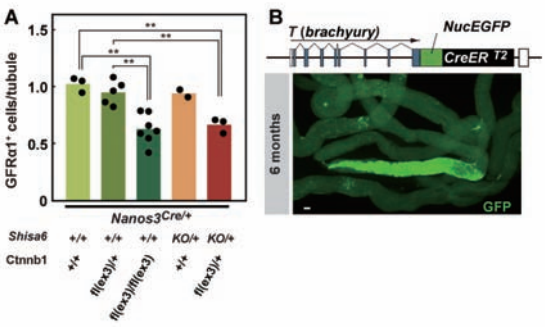


Figure 4. SHISA6 and spermatogenic stem cells (A) SHISA6 maintains the GFR S+ cell pool through inhibiting the Wnt/l pool through i. Average number of GFRve+ cells per tubule section in testes with the indicated genotypes, based on double IF for GFR microtesticular sections. (B) (Upper) A schematic of the structure of the *T*$^{nEGFP\text{-}CreERT2}$ allele, which enables pulse-labeling of T+ cells using CreER^{T2}. (Lower) A result of pulse-labelling of T+ cells with 6 months of chase. Note that GFP (the lineage reporter) positive cells form a prominent patch in a particular segment of seminiferous tubules. Modified from Tokue et al., Stem Cell Reports (2017),

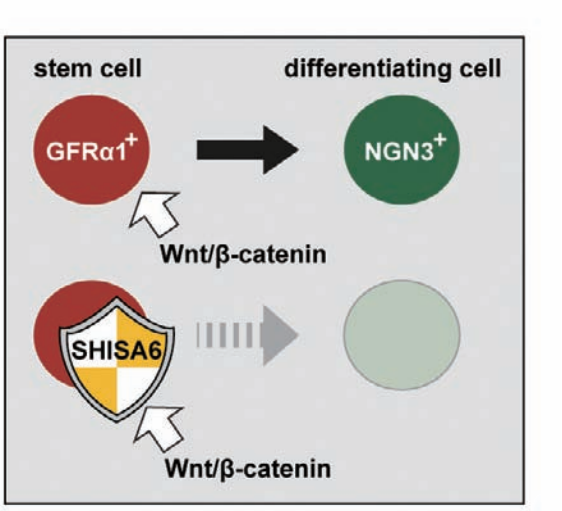


Figure 5. A conceptual diagram of differentiation promotion by Wnt/β-catenin signaling to become Ngn3+ and its cell-autonomous protection by SHISA6 in a subset of GFRd + cells. The heterogeneous expression of SHISA6 may confer different levels of differentiation probability in an open niche environment in which differentiation-promoting Wnt ligand appears to distribute uniformly. Modified from Tokue et al., Stem Cell Reports (2017),

2-4. SHISA6 protects the stem cells from differentiation promoting Wnt/β-catenin signaling: A proposal of a generic mechanism of stem cell regulation in facultative niches

To conclude, we propose a generic mechanism underlying the heterogeneous stem cell fates in facultative niche environments. Different levels of cell-autonomous inhibitor (SHISA6, in this case) may confer heterogeneous resistance to uniformly distributed extracellular signaling that promotes differentiation (such as WNTs). Here, stem cells with higher levels of inhibitors would remain in the undifferentiated cell pool with higher probabilities, while those with lower levels of inhibitors are more inclined to differentiate (Figure 5).

Publication List:

〔Review Article〕

● Yoshida, S. (2016). From cyst to tubule: innovations in vertebrate spermatogenesis. WIREs Developmental Biology *5*, 119-131.

Laboratory of Molecular Genetics for Reproduction †

Associate Professor
TANAKA, Minoru

NIBB Research Fellow:	*NISHIMURA, Toshiya*
Postdoctoral Fellow:	*FUJIMORI, Chika*
SOKENDAI Graduate Student:	*SAKAE, Yuta*
	KIKUCHI, Mariko
	OTAKE, Norihito
Technical Assistant:	*KINOSHITA, Chie*
	WATAKABE, Ikuko
Secretary:	*SHIRAISHI, Naomi*
	FUJISHO, Tamae

Laboratory scope

Reproduction is a universal and fundamental system for organisms to produce new generations. To accomplish this purpose, organisms have developed their own sexual strategies, which allow them to adapt to their environment, thereby progressing toward maximum efficiency of reproduction. During the embryo and larval terms, organisms develop many cell-lineages that have special and essential roles in each different process of reproduction. These lineages are mostly conserved among vertebrates.

Vertebrates, however, exhibit a variety of reproductive systems. The mechanisms of sex determination and sex differentiation are some of the components of the reproductive system which produce this variety. Actually, there are many modes of sex determination. Sex determination genes are different among vertebrates. Sex determination does not even have to be controlled genetically. This variety is allowed by the different employment and different emergence of the cell lineages during embryogenesis. Therefore, it is important to address the roles of each cell lineage for understanding the fundamental mechanism underlying a variety of reproductive systems. Currently, our lab focuses on the core mechanisms which are independent of sex determination genes and which produce and maintain the sex. The core mechanism can be referred to as cellular interaction between germ cells and surrounding somatic cells, wherein germ cells have the ability to feminize somatic cells while the surrounding somatic cells are predisposed to male development. These characters of each set of cells are totally independent of the sex determination gene on the medaka Y chromosome. We are addressing the details of this core mechanism by analyzing each cell lineage in the context of sex differentiation.

To accomplish the purpose of our study, we use medaka fish (*Oryzias latipes*). We have been generating transgenic medaka enabling us to analyze how different cell lineages are involved in the process of gonad formation and sex differentiation *in vivo*. Additionally, in order to identify the genes essential for reproduction, we carried out a mutational screening of medaka with defective phenotypes and disrupted several candidate genes. With these two unique analytical methods (visualizing cells, and mutants), we are attempting to unveil both the fundamental mechanisms and the specific mechanisms that produce a variety of reproductive systems.

†: This laboratory was closed on 31 May, 2016.

Through these analyses, we have been revealing the presence of germline stem cells in the ovary. This was the first proof of this system in vertebrates (Nakamura et al 2010 Science). The fluorescently labeled germline stem cells keep producing eggs with fluorescence during the entire period of medaka reproduction, which is a conclusive indication of the presence of germline stem cells. The tricks employed in this experiment are transgenic medaka that allow heat-inducible gene expression.

I. Identification of a fate switch gene in germ cells (sex determination gene of germ cells)

In the core mechanism of sex, germ cells are responsible for feminization and somatic cells are for masculinization. An important thing here is that we can determine the sex intentionally, if the core mechanism is to be modified, without any effect from the sex determination gene. Then a big issue is how the sex of germ cells, in other words, the fate decision of germ cells to become sperm or eggs, is determined. Few people have addressed this issue in vertebrates.

It is generally accepted that germline stem cells are sexually indifferent or unfixed. And the appearance of germ cells remains the same during the process of both spermatogenesis and oogenesis until germ cells enter meiosis. This suggests that the sex (the fate decision to become eggs or sperm) might not be determined by that time.

Based on these observations, we revealed the genes which differentially express in germ cells just before meiosis. Among them, we successfully identified the gene that determines the sexual fate in germ cells. The gene is one of the genes encoding forkhead transcriptional factors, *foxl3*. The mutant analysis indicated that the factor functions in germ cells autonomously and represses entering spermatogenesis in germ cells. The mutant did not cause any defect in sex differentiation of somatic cells – The XX mutant, genetically female medaka, develops a normal ovary but germ cells develop into sperm in the mutant ovary during young adult period. Artificial insemination with sperm isolated from the mutant ovary and eggs from the wildtype ovary produces fertile eggs, demonstrating that the sperm in the mutant ovary are functional.

II. Structural origins of ovary and testis.

Structural analysis of the mutant ovary leads to one interesting view on the origins of ovary and testis.

The view suggests that indifferent gonads have a common unit for both germinal cradles and lobules. Germinal cradles identify niche regions of germline stem cells in the ovary, and lobules in the testis harbor germline stem cells at their distal ends. Both structures develop from the common unit composed of supporting cells that surround germ cells, which is underlaid by the basement membrane. A mature ovary is composed of both germinal epithelium where germinal cradles (germline niche) are present and the stromal compartment where vitellogenic and/or mature oocytes are present.

Interestingly, the ovary in XX (genetically female) *foxl3* mutant develops a huge expansion of germinal epithelium and sperm is formed in the germinal epithelium. Although the mutant ovary possesses the stromal compartment,

sperm are never observed there. In the expanded area of the germinal epithelium, we can observe common units of germline stem cell-niche, supporting cells with basement membrane. These suggest that the expanded areas within the germinal epithelium are equivalent to testicular lobules.

All the observations mentioned above collectively suggest that testis in the wildtype male can be viewed as the structure organized and developed within the germinal epithelium. In other words it may be possible to assert that mature testis are developed from an origin (a prototype) of germinal epithelium. This is in sharp contrast with the ovary which is composed of germinal epithelium and stromal compartment. Mature eggs are developed in the stromal compartment and the germinal epithelium serves to provide an early stage of diplotene oocytes.

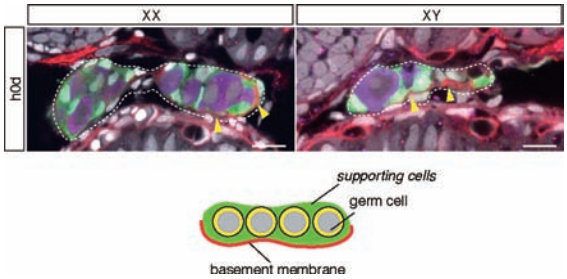


Figure 1. A common unit of ovary and testis. Supporting cells (green) that surround germ cells (yellow) and basement membrane (red) can be viewed as a common unit (below illustration) which develops into the niche structure of ovary (germinal cradles) or of testis (lobules). The unit is observed in both XX and XY gonads (upper images) where yellow arrow heads indicate deposition of basement membrane (red) and supporting cells are stained green. purple: germ cells, white: nuclea

III. Environment during the larval period may affect sex of medaka.

As stated in the beginning, the sex of medaka is genetically determined. The sex determination gene on the Y chromosome determines the male (testicular) fate. However, our result suggests that the sex could be affected by the status of nutrients during the larval stage. If medaka larva is put under food-restricted conditions, the sex ratio of medaka is male-biased. Examination of the developing gonad indicates that the number of typeI germ cells (stem like germ cells) under the restricted conditions are reduced when compared to that under normal conditions. This result may suggest that the sex reversal effect by food restriction may be through germ cell number, which we had reported in previous papers.

Publication List:

〔Review articles〕

- Tanaka, M. (2016). Germline stem cells are critical for sexual fate decision of germ cells. BioEssays *38*, 1227-1233.
- Nishimura, T., Nakamura, S., and Tanaka, M. (2016). A structurally and functionally common unit in testes and ovaries of medaka (*Oryzias latipes*), a teleost fish. Sex. Dev. *10*, 159-165.
- Nishimura, T., and Tanaka, M. (2016). The mechanism of germline sex determination in vertebrates. Biol. Reprod. *95*, 1-6.

DIVISION OF MOLECULAR NEUROBIOLOGY

Professor
NODA, Masaharu

Associate Professor
SHINTANI, Takafumi

Assistant Professor: *SAKUTA, Hiraki*
HIYAMA, Takeshi
Technical Staff: *TAKEUCHI, Yasushi*
NIBB Research Fellow: *NOMURA, Kengo*
Postdoctoral Fellow: *FUJIKAWA, Akihiro*
SUZUKI, Ryoko
KUBOYAMA, Kazuya
LIN, Chia-Hao
SOKENDAI Graduate Student:
MATSUDA, Takashi
YU, Yang
TANGA, Naomi
HIGASHI, Satoru
Technical Assistant: *MIURA, Seiko*
NAKANISHI, Norie
DOKYO, Yumi
WADA, Kotoe
KONISHI, Mie
ISOSHIMA, Yoshiko
HASHIMOTO, Terumi
Secretary: *KODAMA, Akiko*

We have been studying the molecular and cellular mechanisms underlying the development of the vertebrate central nervous system (CNS), mainly using mice. This research covers many developmental events including the patterning of the retina, neuronal terminal differentiation, axonal navigation, branching and targeting, synapse formation, refinement and plasticity. The scope of our interests also encompasses the mechanisms for various functions of the mature brain, including body-fluid regulation, behavior control, learning, and memory.

I. Mechanisms for neural circuit formation

Adenomatous polyposis coli 2 (APC2) is preferentially expressed in the nervous system from early developmental stages through to adulthood. APC2 is distributed along actin fibers as well as microtubules in neurons. Our investigation suggests that APC2 is involved in the signaling pathway from membrane receptors for extracellular guidance factors to the intracellular migration machinery.

Sotos syndrome (OMIM #117550) is characterized by intellectual disability and a combination of typical facial features and large head circumference. Sotos syndrome has been known to be caused by haploinsufficiency in the *NSD1* gene. Our knockdown experiments revealed that the expression of *APC2* in the nervous system was under the control of *NSD1*. Moreover, *Apc2*-knockout (KO) mice also showed Sotos syndrome-like abnormalities. We are now investigating the relationship between NSD1 and APC2 in more detail by examining *Nsd1*-KO mice.

II. Physiological roles of receptor-like protein tyrosine phosphatases

Protein tyrosine phosphorylation plays crucial roles in various biological events such as cellular proliferation, differentiation, survival, migration, and metabolism. Cellular tyrosine phosphorylation levels are governed by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). The physiological functions and regulatory mechanisms of receptor-like PTPs (RPTPs) are not fully elucidated. We have been making efforts to reveal the functional roles of RPTPs, especially of the R3 and R5 subfamilies.

2-1 R3 RPTP subfamily

The R3 RPTP subfamily, which is comprised of PTPRB, PTPRH, PTPRJ, and PTPRO, reportedly plays pivotal roles in the development of several tissues including the vascular and nervous systems. We demonstrated, for the first time, that PTPRJ is a physiological enzyme attenuating insulin signaling *in vivo*. *Ptprj*-KO mice show lower weight gain associated with lesser food intake compared with wild-type mice. Thus we are now investigating a role of PTPRJ in the regulation of energy homeostasis.

2-2 R5 RPTP subfamily

PTPRZ is the most abundant RPTP in oligodendrocyte precursor cells (OPCs), which are the principal source of myelinating oligodendrocytes. Three PTPRZ isoforms are generated by alternative splicing from a single *PTPRZ* gene: two transmembrane isoforms, PTPRZ-A and PTPRZ-B, and one secretory isoform, PTPRZ-S, all of which expressed in the CNS are heavily modified with chondroitin sulfate (CS) chains. The CS moiety on their extracellular domain of PTPRZ is essential for achieving high-affinity binding sites for the endogenous ligands; pleiotrophin (PTN), midkine, and interleukin-34. However, its functional significance and molecular mechanism in regulating PTPRZ activity remains obscure.

We have shown that PTPRZ receptors have functions to maintain the immature status of OPCs by dephosphorylating various substrate molecules including p190 RhoGAP. This year, we revealed that protein expression of CS-modified PTPRZ-A began earlier, peaking at postnatal days 5–10 (P5–P10), and then that of PTN peaked at P10 at the developmental stage corresponding to myelination onset in the mouse brain. *Ptn*-KO mice consistently showed a later onset of the expression of myelin basic protein, a major component of the myelin sheath, than wild-type mice. Upon ligand application, PTPRZ-A/B in cultured oligodendrocyte precursor cells exhibited punctate localization on the cell surface instead of diffuse distribution, causing the inactivation of PTPRZ and oligodendrocyte differentiation. Notably, the same effect was observed with the removal of CS chains with chondroitinase ABC (chABC), but not polyclonal antibodies against the extracellular domain of PTPRZ (Figure 1). These data indicate that PTN-PTPRZ-A signaling controls the timing of oligodendrocyte precursor cell differentiation *in vivo*, in which the negatively charged CS moiety maintains PTPRZ in a monomeric active state, and that the positively-charged PTN induces receptor clustering, potentially by neutralizing electrostatic repulsion between CS chain.

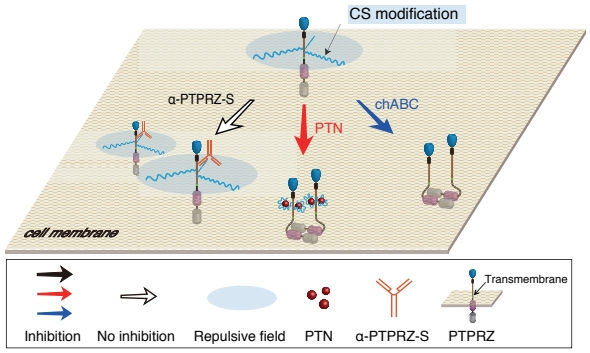


Figure 1. Schematic representation of the role of the CS chains in regulating PTPRZ activity. The CS modification in PTPRZ was essential for maintaining the monomeric active form, possibly by electrostatic repulsion, and, at the same time, provided ligand binding sites. The binding of PTN appears to neutralize the negative charges of the CS chains, causing the dimerization and inactivation of PTPase (see text).

Emerging data have indicated that PTPRZ is aberrantly over-expressed in glioblastoma and it is a causative factor for its malignancy. However, small molecules that selectively inhibit the catalytic activity of PTPRZ have not been developed so far. We identified SCB4380 as the first potent inhibitor for PTPRZ by *in vitro* screening of a chemical library. We determined the crystal structure of the catalytic domain of PTPRZ. Furthermore, the structural basis of the binding of SCB4380 elucidated by a molecular docking method was validated by site-directed mutagenesis studies (Figure 2A). The intracellular delivery of SCB4380 by liposome carriers inhibited PTPRZ activity in C6 glioblastoma cells, and thereby suppressed their migration and proliferation *in vitro* and tumor growth in a rat allograft model (Figure 2B). Therefore, selective inhibition of PTPRZ represents a promising approach for glioma therapy.

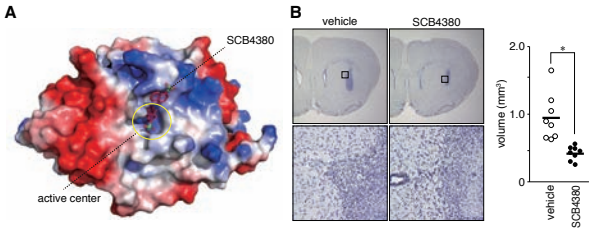


Figure 2. Development of SCB4380 and its application to a rat glioblastoma model. **A**, whole view of the best docking pose of SCB4380 shown by stick representation. **B**, rat C6 glioblastoma cells transplanted into rat brains were allowed to grow for 7 days. One day after surgery, SCB4380/liposome, or vehicle was icv injected daily for 5 days. Representative sections are shown together with an enlargement of the boxed area. The graph shows tumor volume. The horizontal bars indicate the average of each group. $*P<0.05$, by the Student's *t*-test.

III. Brain systems for body-fluid homeostasis

We have previously shown that Na_x, which structurally resembles voltage-gated sodium channels ($Na_v1.1$–1.9), is a Na^+-concentration ($[Na^+]$)-sensitive Na channel with a gating threshold of ~150 mM for extracellular $[Na^+]$ ($[Na^+]_o$) *in vitro*. Na_x is preferentially expressed in the glial cells of sensory circumventricular organs (sCVOs) including the subfornical organ (SFO) and organum vasculosum lamina terminalis (OVLT), and is involved in $[Na^+]$ sensing for the control of salt-appetite.

3-1 Thirst control in the brain by Na_x and TRPV4

We found that Na_x is also involved in thirst control in dehydrated animals (Figure 3). We investigated voluntary water intake immediately induced after an intracerebroventricular (icv) administration of a hypertonic NaCl solution in mice. Because the transient receptor potential vanilloid (TRPV) channels, TRPV1 and TRPV4, have been proposed to function as osmosensors, we examined *TRPV1*-, *TRPV4*-, *Na_x*-, and their double-gene KO mice. The induction of water intake by *TRPV1*-KO mice was normal, whereas that by *TRPV4*-KO and *Na_x*-KO mice was significantly less than that by WT mice. Water intake by *Na_x*/*TRPV4*-double KO mice was similar to that by the respective single KO mice. When TRPV4 activity was blocked with a specific antagonist (HC-067047), water intake by WT mice was significantly reduced, whereas that by *TRPV4*-KO and *Na_x*-KO mice was not. The same results were obtained by the administration of miconazole, which inhibits the biosynthesis of epoxyeicosatrienoic acids (EETs), endogenous agonists for TRPV4, from arachidonic acid (AA). Moreover, icv injection of hypertonic NaCl with AA or 5,6-EET restored water intake by *Na_x*-KO mice to the WT level, but not that by *TRPV4*-KO mice. These results suggest that the Na^+ signal generated in Na_x-positive glial cells leads to the activation of TRPV4-positive neurons in sCVOs in order to stimulate water intake by using EETs as gliotransmitters.

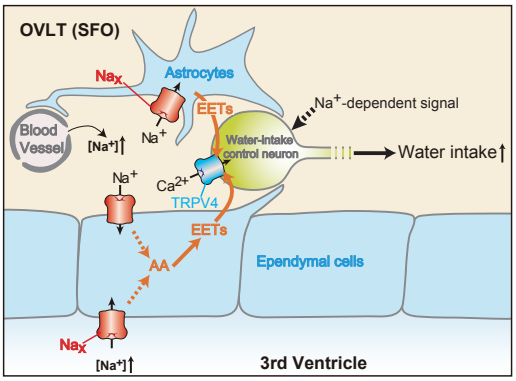


Figure 3. A possible signaling pathway in sCVOs underlying the induction of water intake. When $[Na^+]_o$ in plasma and cerebrospinal fluid increases, Na_x channels (red) in glial cells (astrocytes and ependymal cells) in the SFO or OVLT are activated, leading to the synthesis of EETs. EETs released from glial cells activate TRPV4 channels (blue) in the neighboring neurons, which conceivably control water-intake behavior. The additional activation signal of an unknown Na^+-dependent mechanism is required for the Na_x/TRPV4-dependent induction of water intake.

3-2 Thirst and salt-appetite control in the brain by angiotensin II, $[Na^+]$, and cholecystokinin

Angiotensin II (Ang II) is known to drive both thirst and salt appetite; however, the neural mechanisms underlying selective water- and/or salt-intake behaviors remain unknown. Using optogenetics, we showed that thirst and salt appetite are driven by distinct groups of angiotensin

II receptor type 1a-positive excitatory neurons in the SFO (Figure 4). Neurons projecting to the OVLT control water intake, while those projecting to the ventral part of the bed nucleus of the stria terminalis (vBNST) control salt intake. Thirst-driving neurons are suppressed under sodium-depleted conditions through cholecystokinin (CCK)-mediated activation of GABAergic neurons. In contrast, the salt-appetite-driving neurons were suppressed under dehydrated conditions through activation of another population of GABAergic neurons by Na_x signals. These distinct mechanisms in the SFO may underlie the selective intakes of water and/or salt and may contribute to body fluid homeostasis.

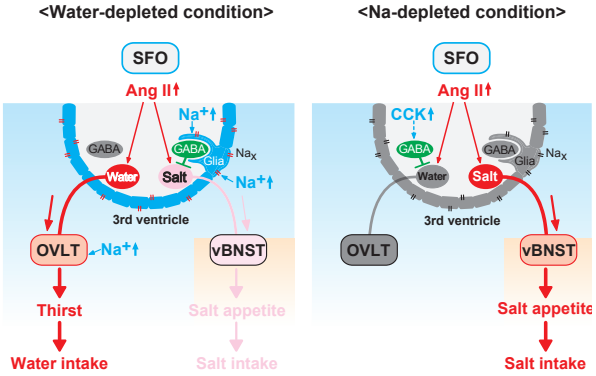


Figure 4. A schematic overview of controls for thirst and salt appetite from SFO. Under the water-depleted condition (left), both Ang II and [Na^+] increase in SFO. "Water neurons" innervating OVLT are selectively activated under the water-depleted condition. Under the Na-depleted condition (right), both Ang II and CCK increase in SFO, and "salt neurons" which innervate vBNST are selectively activated. Under the water- and Na-depleted condition, both water and Na intakes are stimulated, because neither the Na_x signal nor CCK signal is induced.

3-3 Adipsic hypernatremia accompanied by autoantibodies to the SFO

Adipsic (or essential) hypernatremia is a rare hypernatremia caused by a deficiency in thirst regulation and vasopressin release. In 2010, we reported a case in which autoantibodies targeting Na_x caused adipsic hypernatremia without hypothalamic structural lesions demonstrable by magnetic resonance imaging (MRI). This year, we reported three newly identified patients with similar symptoms. Immunohistochemical analyses using the sera of these patients revealed that antibodies specifically reactive to the mouse SFO were present in the sera of all cases. Passive transfer to mice of the patient immunoglobulin revealed that autoimmune destruction of the SFO may be the cause of the adipsic hypernatremia. This study provides a possible explanation for the pathogenesis of adipsic hypernatremia without demonstrable hypothalamus-pituitary lesions.

Publication List:

〔Original papers〕

- Doura, T., Kamiya, M., Obata, F., Yamaguchi, Y., Hiyama, T.Y., Matsuda, T., Fukamizu, A., Noda, M., Miura, M., and Urano, Y. (2016). Detection of *lacZ*-positive cells in living tissue with single-cell resolution. Angew. Chem. Int. Ed. Engl. *55*, 9620–9624.
- Fujikawa, A., Nagahira, A., Sugawara, H., Ishii, K., Imajo, S., Matsumoto, M., Kuboyama, K., Suzuki, R., Tanga, N., Noda, M., Uchiyama, S., Tomoo, T., Ogata, A., Masumura, M., and Noda, M. (2016). Small-molecule inhibition of PTPRZ reduces tumor growth in a rat model of Glioblastoma. Sci. Rep. *6*, 20473.
- Kuboyama, K., Fujikawa, A., Suzuki, R., Tanga, N., and Noda, M. (2016). Role of chondroitin sulfate (CS) modification in the regulation of protein tyrosine phosphatase receptor type Z (PTPRZ) activity: Pleiotrophin-PTPRZ-A signaling is involved in oligodendrocyte differentiation. J. Biol. Chem. *291*, 18117-18128.
- Sakuta, H., Nishihara, E., Hiyama, T.Y., Lin, C.-H., and Noda, M. (2016). Na_x signaling evoked by an increase in [Na^+] in CSF induces water intake via EET-mediated TRPV4 activation. Am. J. Physiol. Regul. Integr. Comp. Physiol. *311*, R299-R306.

〔Original papers (E-publication ahead of print)〕

- Hiyama, T.Y., Utsunomiya, A.N., Matsumoto, M., Fujikawa, A., Lin, C.-H., Hara, K., Kagawa, R., Okada, S., Kobayashi, M., Ishikawa, M., Anzo, M., Cho, H., Takayasu, S., Nigawara, T., Daimon, M., Sato, T., Terui, K., Ito, E., and Noda, M. Adipsic hypernatremia without hypothalamic lesions accompanied by autoantibodies to subfornical organ. Brain Pathol. 2016 Aug 2.
- Matsuda, T., Hiyama, T.Y., Niimura, F., Matsusaka, T., Fukamizu, A., Kobayashi, K., Kobayashi, K., and Noda, M. Distinct neural mechanisms for the control of thirst and salt appetite in the subfornical organ. Nat. Neurosci. 2016 Dec 19.

〔Review articles〕

- Fujikawa, A., and Noda, M. (2016). Role of pleiotrophin-protein tyrosine phosphatase receptor type Z signaling in myelination. Neural Regen. Res. *11*, 549-551.
- Hiyama, T.Y., and Noda, M. (2016). Sodium sensing in the subfornical organ and body-fluid homeostasis. Neurosci. Res. *113*, 1-11.

DIVISION OF BEHAVIORAL NEUROBIOLOGY

Professor
HIGASHIJIMA, Shin-ichi

Assistant Professor: *KIMURA, Yukiko*
Postdoctoral Fellow: *KIMURA, Yukiko**
Visiting Graduate Student: *SHIMAZAKI, Takashi*
UEMURA, Yuto
Technical Assistant: *SUZUKI, Sachiko*
TERASAWA, Yoko
WATAKABE, Ikuko
ITO, Hiroko
Secretary: *TAKEUCHI, Yoshiko*

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.

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).

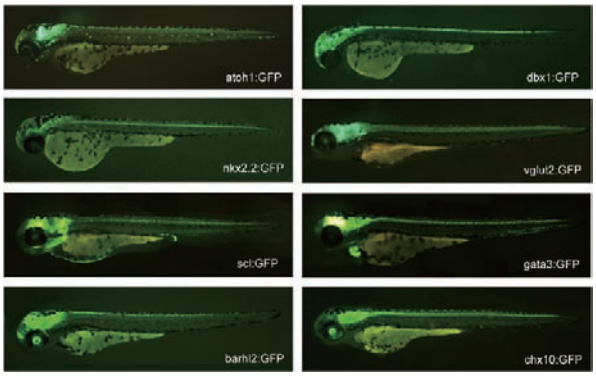


Figure 1. Examples of transgenic fish expressing GFP in specific classes of neurons.

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 inhibition 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.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2016. The former title is indicated by an asterisk (*).

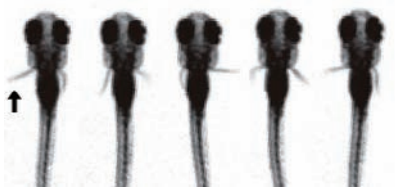


Figure 2. Rhythmic movements of the pectoral fin (arrow) during swimming in larval zebrafish.

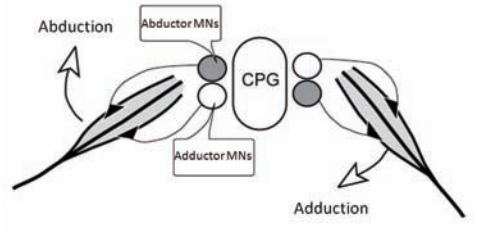


Figure 3. Schematic of rhythmic movements of pectoral fins during swimming. CPG, Central Pattern Generator.

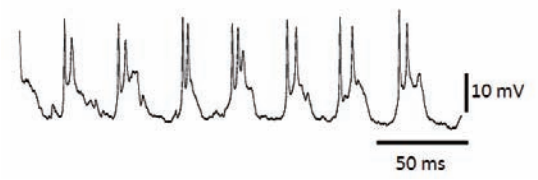


Figure 4. Rhythmic firings of an abductor motoneuron during swimming.

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.

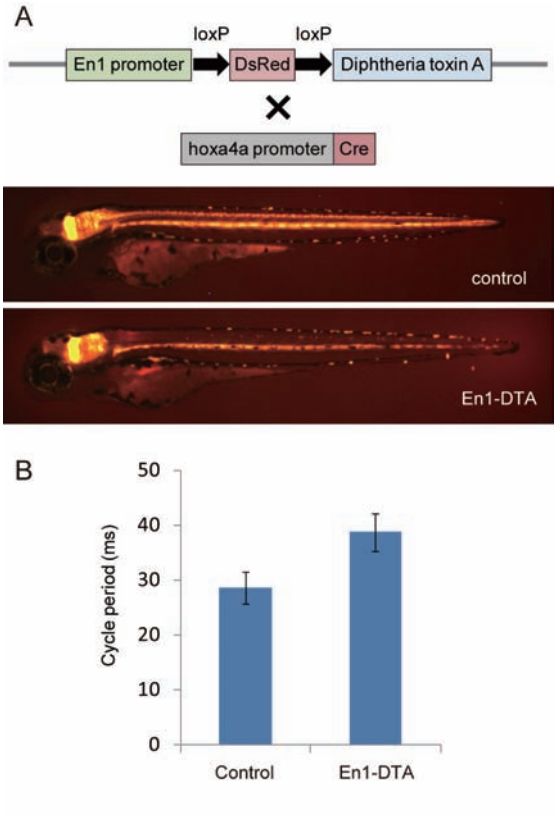


Figure 5. Spinal En1 neurons play an important role for controlling swimming speed. A, Ablation of spinal En1 neurons by using the Cre-loxP system. DTA, diphtheria-toxin A. B, Cycle period of the motor bursts during regular swimming in control and En1-DTA larvae.

Publication List:

〔Original Papers〕

- Chou, M.-Y., Amo, R., Kinoshita, M., Cherng, B.-W., Shimazaki, H., Agetsuma, M., Shiraki, T., Aoki, T., Yamazaki, M., Higashijima, S., and Okamoto, H. (2016). Social conflict resolution regulated by two dorsal habenular subregions in zebrafish. Science *352*, 87-90.
- Ota, S., Taimatsu, K., Yanagi, K., Namiki, T., Ohga, R., Higashijima, S., and Kawahara, A. (2016). Functional visualization and disruption of targeted gene using CRISPR/Cas9-mediated eGFP reporter integration in zebrafish. Sci. Rep. *6*, 34991.

DIVISION OF BRAIN CIRCUITS †

Professor
MATSUZAKI, Masanori

Assistant Professor:	*HIRA, Riichiro*
	MASAMIZU, Yoshito
Technical Staff:	*OHSAWA, Sonoko*
NIBB Research Fellow:	*EBINA, Teppei*
Postdoctoral Fellow:	*TANAKA, Yasuhiro*
	TANAKA, Yasuyo
	KONDO, Masashi
	SHINOTSUKA, Takanori
	TAKEDA, Yuta
SOKENDAI Graduate Student:	*OHKUBO, Fuki*
	HASEGAWA, Ryota
	SUZUKI, Ayami
Visiting Graduate Student:	*TERADA, Shin-Ichiro*
Visiting Scientist:	*HIRAKAWA, Reiko*
Technical Assistant:	*ATSUMI, Urumi*
	OHARA, Kaori
	KOTANI, Keiko
	TAKAHASHI, Yoichi
	IWASE, Etsuko
	IMOTO, Eiko
Secretary:	*SUGIYAMA, Tomomi*

Various firing patterns of many cortical neurons represent information processing in the brain. The microarchitecture of synaptic connections control information processing in cortical circuits. The structure and location of synapses determine and modify the strength of this information processing. The aim of our laboratory is to reveal how information is formed, maintained, selected, and decoded in the brain at the levels of single cells and of single synapses. To do so, we mainly use two-photon microscopy that allows us to see fluorescence signals from deep within living tissue, developing novel photostimulation methods and animal behavioral tasks. The goals of our recent studies are to reveal how voluntary movement is memorized and represented in cortical circuits. In addition, we are working to apply two-photon microscopy to a non-human primate, the common marmoset, in order to understand information processing in the brain, which is relevant to high cognitive functions.

I. Measurement of thalamocortical activities in the primary motor cortex during voluntary movements.

The primary motor cortex (M1) integrates a variety of information from other brain regions and transmits motor commands to the spinal cord. Information from the basal ganglia and cerebellum, which is thought to be critical for motor learning and motor execution, is signaled to the M1 through the thalamus. However, little is known about the pattern of thalamocortical activities in the M1 during self-initiated voluntary movements. Thus, we have examined activity patterns in layer 1 (L1) and 3 (L3) thalamocortical axons in the M1, which presumably receive information from the basal ganglia and cerebellum, respectively. We conducted two-photon calcium imaging of L1 and L3 thalamocortical axons in the mouse forelimb M1 during a lever pull task (Hira et al., 2013; Masamizu et al., 2014). We found that the timing of peak activity in L1 thalamocortical axons was sequentially distributed as a population during lever-pull movement. By contrast, the peak and onset timings of the activity in L3 thalamocortical axons were mainly seen at lever pull initiation. During learning, the population of L1 TC axonal boutons obtained sparse lever-relevant neural code, while the population of L3 TC axonal boutons evolved to be robust. Optogenetic experiments showed that the thalamocortical axons have the ability to induce a forelimb movement and modify lever pull movement. Our results indicate that L1 and L3 thalamocortical activities convey distinct information required for learning of voluntary movements.

The axon imaging described above is much more vulnerable to the motion of the imaging plane than imaging of cell bodies because of the small diameters (less than 1 μm) of axons. Especially, motion in the normal direction to the imaging plane (z-motion) often causes artefactual changes in fluorescence intensity of axons. These changes can be confounding in interpreting fluorescence transients observed in axons of awake active animals. To solve this problem, we developed a novel registration method with z-motion correction for axon imaging. In the mouse motor cortex, four-dimensional images of axons expressing fluorescent protein were obtained with two-photon microscopy equipped with a piezoelectric objective lens positioning system. Then, the imaging data were analyzed off-line. At every time point, two of three z-planes were blended at an appropriate ratio to obtain a composite plane similar to the target, the temporal average image of second z-planes. The x-y displacements of two blended images and their blending ratio were determined using a particle filter. In the particle filter, each of three parameters decomposed to two system variables, one obeying a second-order trend model and another a third-order auto-regressive model and system noise was generated from Gaussian or Cauchy distribution. The resample weight for each particle was calculated with the correlation between each composite image and the target. The fluorescence intensity of axons in obtained time series of composite planes was significantly less deviated than that in original images. By using this method, we were able to analyze the axonal imaging data.

II. Wide-field two-photon microscopy for simultaneous imaging of multiple cortical areas at cellular resolution

Understanding the dynamics of cortico-cortical communications is essential because information processing in the brain is not only performed in intra-areal circuits but also through inter-areal interactions. To execute voluntary movements, the interactions between M1 and the secondary motor area (M2) and between M1 and sensory-associated areas are crucial. Recent advances in two-photon microscopy have allowed us to image a relatively large area (up to 1 mm) at cellular resolution. However, it is still difficult to image a continuous large field (>2 mm) and two distant (>3 mm) brain areas at cellular resolution using a single two-photon microscope. We developed super-wide-field two-photon microscopy with

†: This laboratory was closed on 31 March, 2016.

a single objective, which allows the imaging of two distant (up to 6 mm apart) cortical areas and a large continuous area (up to 3 mm) at cellular resolution. The method depends on placing a novel optical device under a high-NA objective with a long working distance in a standard two-photon microscope. The device is composed of a pair of mirrors and a holder to rotate the mirrors perpendicular to the optical axis. By controlling the rotation timing and angle using custom-made software, the field of view can be rapidly switched without moving either the objective or the sample.

By rotating the mirror pair back and forth between two angles, we conducted sequential two-photon calcium imaging of neuronal activities in two distant areas up to 6 mm apart and at a depth of up to 800 μm from the cortical surface. Furthermore, by stitching the fields of view, we succeeded in imaging a 3 mm × 1 mm continuous area. We applied it to concurrent calcium imaging of layer 2/3 and layer 5 neurons in rostral (M2) and caudal (M1) motor cortical areas while the mice performed a lever-pull task. We are analyzing how the neural activities in these fields are coordinated during motor execution. Importantly, the optical device together with the controller can be easily installed on a standard two-photon microscope. Its adaptation by neuroscientists should open the door to the study of information processing in brain networks at cellular resolution.

III. Marmoset forelimb-movement tasks for two-photon Ca^{2+} imaging of the motor cortex

Recent advances in calcium imaging have revealed cellular and subcellular mechanisms underlying a variety of brain functions in rodents, fishes, and invertebrates. However, the calcium imaging technique is still difficult to apply to awake non-human primates, especially during forelimb movement tasks, which are very useful for investigating the mechanisms underlying cognitive behaviors, decision making, motor planning/execution and motor skill learning. We have extended the technique of two-photon calcium imaging, which we established in a non-human primate, the common marmoset, in an anesthetized state (Sadakane et al., 2015), to record neuronal activity in the cerebral cortex of behaving marmosets.

To do so, we developed a novel lever-manipulation device with a chair that restrains the head and body, but not forelimbs. Three marmosets were trained with the device 10–60 min per day for 2–3 weeks. The marmosets successfully learned a self-initiated lever-pull task, in which the animals have to pull the lever to get a reward. The marmosets also learned a visual cued lever-pull task, in which the reward is delivered only when the cue is presented on the monitor and the lever is pulled, with additional 1–2 weeks of the training. We conducted two-photon calcium imaging in M1 of the marmosets during the task, using novel marmoset-specific two-photon microscopy, which allows us to observe any dorsal cortical area in marmosets sitting on the chair. We detected calcium transients responding to forelimb movements from somata and dendrites.

Overall, these results indicate that forelimb movement tasks for head-restrained marmosets are feasible and that neural activities can be monitored in the neocortex of behaving marmosets with cellular and subcellular resolution over days by two-photon calcium imaging.

Publication List:

〔Original paper〕

- Terada, S., Matsubara, D., Onodera, K., Matsuzaki, M., Uemura, T., and Usui, T. (2016). Neuronal processing of noxious thermal stimuli mediated by dendritic Ca^{2+} influx in *Drosophila* sensory neurons. eLife *5*, e12959.

LABORATORY OF NEUROPHYSIOLOGY

Associate Professor
WATANABE, Eiji

NIBB Research Fellow: *YASUGI, Masaki*

In order to interact successfully with the environment, animals must deduce their surroundings based on sensory information. The visual system plays a particularly critical role in such interactions with the environment.

"Why can we see?" This question is fundamental for a thorough understanding of vision-dependent animals, including human beings. In order to better understand the visual system of animals, we are researching animal behaviors through psychophysical and computational methods.

I. Psychophysical study of Medaka fish

One of our major subjects is the psychophysical and computational study of medaka (*Oryzias latipes*). Recently, we made progress in studies of prey-predator interaction using medaka and zooplankton. Visual motion cues are one of the most important factors for eliciting animal behavior, including predator-prey interactions in aquatic environments. To understand the elements of motion that cause such selective predation behavior, we used a virtual plankton system where the predation behavior in response to computer-generated prey was analyzed. Virtual prey models were programmed on a computer and presented to medaka, which served as predatory fish. Medaka exhibited predation behavior against several characteristic virtual plankton movements, particularly against a swimming pattern that could be characterized as pink noise motion. Analyzing prey-predator interactions via pink noise motion will be an interesting research field in the future (Matsunaga & Watanabe, 2012).

In recent years, we have made progress in studies of the schooling behaviors of medaka. Many fish species are known to live in groups. Visual cues have been shown to play a crucial role in the formation of shoals. Using biological motion stimuli, depicting a moving creature by means of just a few isolated points, we examined whether physical motion information is involved in the induction of shoaling behavior. We found that the presentation of virtual biological motion can prominently induce shoaling behavior. We have shown what aspects of this motion are critical in the induction of shoaling behavior. Motion and behavioral characteristics can be valuable in recognizing animal species, sex, and group members. Studies using biological motion stimuli will enhance our understanding of how non-human animals extract and process information which is vital for their survival (Nakayasu & Watanabe, 2014).

We have developed a novel method for behavior analysis using 3D computer graphics. The fine control of various features of living fish have been difficult to achieve in studies of fish behavior. However, computer graphics allow us to manipulate morphological and motion cues systematically. Therefore, we have constructed 3D computer graphic animations of medaka based on tracking coordinate data and photo data obtained from real medaka. These virtual 3D models will allow us to represent medaka faithfully and to undertake a more detailed analysis of the properties of the visual stimuli that are critical for the induction of various behaviors. This year, we began studying artificial intelligence (AI) programmed virtual 3D models (Figure 1). The AI models will lead us in future work on animal behaviors.

Simultaneously, we began studying "behavioral lateralization" as a characteristic which can affect the formation of shoals. Like humans, fishes have lateral differences in their movement and perception. However, there have been few studies to examine the influence of behavioral lateralization on their interaction. If each individual has a laterally biased response to surrounding companions or predators, how is the united movement as shoals realized? This study is expected to bring a new viewpoint for understanding the structure and behavior of schooling.

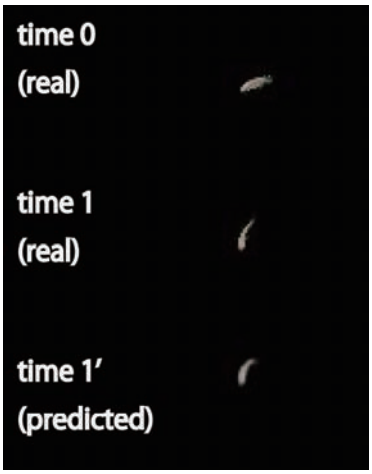


Figure 1. Unsupervised learning of video representations using a deep learning method (LSTMs). The AI predicts fish position at time 1 in reference to fish position at time 0.

II. Psychophysical study of Human vision

Another of our major subjects is the psychophysical and theoretical studies of the visual illusions experienced by human beings (*Homo sapiens*). One recent focus of this debate is the flash-lag effect, in which a moving object is perceived to lead a flashed object when both objects are aligned in actual physical space. We developed a simple conceptual model explaining the flash-lag effect. In recent years, we have made more developed novel visual illusions, such as the shelf-shadow illusion .

Publication List:

〔Original papers〕

● Hoshino, A., et al. (2016). Genome sequence and analysis of the Japanese morning glory *Ipomoea nil*. Nat. Commun. *7*, 13295.
● Yasugi, M., and Hori, M. (2016). Predominance of parallel- and cross-predation in anglerfish. Marine Ecol. *37*, 576-587.

DIVISION OF EVOLUTIONARY BIOLOGY

Professor
HASEBE, Mitsuyasu

Associate Professor
MURATA, Takashi

Assistant Professor:	*TAMADA, Yosuke*
	ISHIKAWA, Masaki
Technical Staff:	*KABEYA, Yukiko*
NIBB Research Fellow:	*IMAI, Akihiro*
Postdoctoral Fellow:	*MANO, Hiroaki*
	TORIBA, Taiyo
SOKENDAI Graduate Student:	*KAMIDA, Chiharu*
	KOSHIMIZU, Shizuka
	LI, Chen
	MORISHITA, Mio
	PALFALVI, Gergo
	SUDA, Hiraku
	SUGAYA, Tomomi
	ZHANG, Liechi
	HORIUCHI, Yuta
	DE VILLIERS, Ruan
Visiting Graduate Student:	*GU, Nan*
Visiting Undergraduate:	*SAKAZAKI, Takuya*
Technical Assistant:	*AOKI, Etsuko*
	GOTO, Misako
	HIRAMATSU, Mika
	KAJIKAWA, Ikumi
	MORI, Asuka
	MATSUZAKI, Yoko
	NISHI, Tayo
	OOI, Shoko
Secretary:	*KOJIMA, Yoko*

I. Evolution of Complex Adaptive Characters

The theory of natural selection and the neutral theory of molecular evolution are powerful concepts in evolutionary biology. However, even with such theories, there still remain unexplained phenomena, one of which is the evolution of complexity. It is difficult to explain the mechanisms needed to evolve complex adaptive traits at cellular and organismal levels, such as cell division machinery, regeneration, novel organ development, host race change, and mimicry. Such traits comprise many components and become adaptive only when all components are gathered together. However, based on evolutionary theory, each component should evolve one by one according to the accumulation of mutations. We aim to reveal the genetic networks regulating these complex traits and to infer the mechanisms needed to evolve complex characters.

II. Spatiotemporal regulation of cell division axis as a grand plan of plant developmental evolution

Cell division axis has to be properly regulated during development in both metazoans and land plants. Genetic changes in the regulation of cell division axis lead to the development of multicellular organisms. Since land plants do not have centrosomes and asteroid bodies, both of which are involved in the axis formation of metazoans, land plants should have different regulatory mechanisms. We aim to investigate the connecting factors between microtubules and GRAS transcription factors that regulate periclinal cell divisions in the moss *Physcomitrella patens*. In addition to identify the factors, the spatiotemporal regulatory mechanisms will be studies to understand the basis of body plan evolution with comparison to those in the flowering plant *Arabidopsis thaliana* and the green algae *Closterium peracerosum–strigosum-littorale*. This is a collabortion project between our division and Dr. Rumiko Kofuji in Kanazawa University, Dr. Hiroyuki Sekimoto in Japan Women's University, and Atsushi Mochizuki in RIKEN.

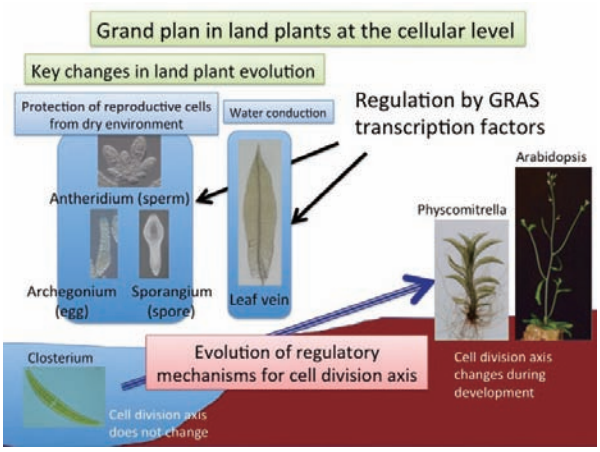


Figure 1. Evolution of the regulatory mechanisms for cell division axis appears to be the basic change leading to the subsequent divergence of land plants.

III. Evolution of Elaborated Cell Division Machinery: Spindle body

At mitosis, all eukaryotic cells divide chromosomes to two daughter cells using a bipolar mitotic spindle, which is composed of microtubules. The centrosomes, which act as microtubule organizing centers, induce formation of the two poles in metazoan cells. In contrast, the cells of land plants and their sister group, zygnematales green algae, form the bipolar spindle in the absence of centrosomes. For understanding the mechanism of acentrosomal spindle formation, the steps of microtubule reorganization during spindle formation should be visualized. We collaborated with Prof. Tomomi Nemoto in Hokkaido University and developed a two-photon spinning disk confocal microscope, which enables 3-dimensional imaging of living cells with high temporal and spatial resolution. We found that spindle microtubules elongate from a prospindle, that is, a microtubule cage with two poles on the nuclear envelope. Our data suggest that the prospindle organizes the bipolar spindle, as centrosomes do in metazoan cells. In contrast to the metazoan centrosomes, however, the prospindle disappears before metaphase. To understand the mechanism how the bipolar spindle is maintained in the absence of the organizer, we established a minispindle system, which involves a bipolar microtubule complex composed of an isolated chromosome and microtubules in tobacco cells. Analyses of microtubule behavior in the minispindle are in progress. Takashi Murata is a main researcher of this study.

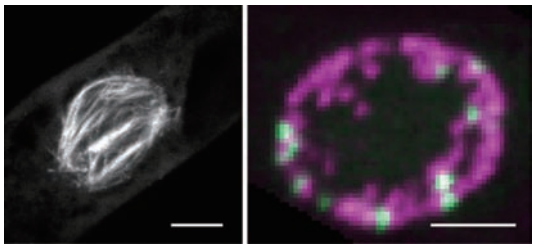


Figure 2. Microtubules (left) and chromosomes (right) during spindle formation of tobacco BY-2 cells. In the right panel, Chromosomes and centromeres are shown as magenta and green, respectively. Bar, 10 μm.

IV. Evolution of Regeneration: Reprogramming of Differentiated Cells to Pluripotent Stem Cells

Different species have different morphology and also cellular characters vary between species. Stem cells self-renew and repeatedly produce differentiated cells during development. Conversely, differentiated cells can be converted into stem cells in some organisms. In plants, the reprogramming to a stem cell can lead to generation of a new individual, which is an effective strategy for propagation. The ability to reprogram is different from species to species but the reason is unknown. The moss *Physcomitrella patens* has a rapid reprogramming ability and is feasible for use in experiments. Cells in a dissected leaf are reprogrammed to become chloronema apical stem cells within 24 hours. We found that *P. patens COLD SHOCK DOMAIN PROTEIN* genes (*PpCSP*s) are positive regulators of the reprogramming in Physcomitrella. Stem-cell formation was enhanced in the over-expression lines of a *PpCSP* gene (Figure 3). Quadruple deletion mutants of *PpCSPs* exhibited attenuated reprogramming. PpCSPs share conserved domains with an induced pluripotent stem (iPS) cell factor Lin28 in mammals, indicating that closely related proteins function in the enhancement of reprogramming in both land plant and metazoan lineages. This research is mainly performed by Chen Li and Yosuke Tamada.

The stem cell formation requires light and wounding signals. We found that the stem cell formation was facilitated in the quadruple deletion mutant of the *P. patens SQUAMOSA promoter binding protein* (*PpSBP*) genes, some of which are known to be repressed by light signals. In addition, we found that *PpSBPs* are negatively regulated by the wounding signal. Characterization of *PpSBPs* are in progress mainly by Yukiko Kabeya and Yosuke Tamada to investigate cross talk between light and wounding signaling pathways in the process of stem cell formation.

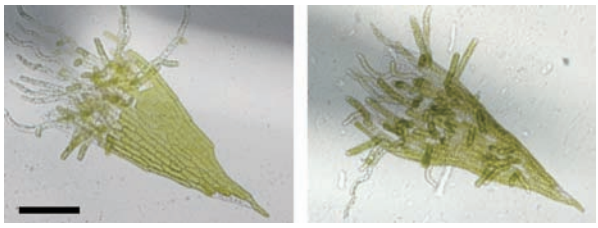


Figure 3. Leaves of wild type (left) and *PpCSP1* Bar, 200 μm.

V. Evolution of Regeneration: Master Regulator for Reprogramming *STEMIN*

Animal somatic cells can be reprogrammed to iPS cells by introducing four transcription factors, while such factors have not been identified in plants. We have previously identified a gene encoding a member of a plant-specific transcription factor, STEM CELL-INDUCING FACTOR 1 (STEMIN1) that was able to induce direct reprogramming of differentiated leaf cells into chloronema apical stem cells without wounding signals. *STEMIN1* and its two paralogous genes (*STEMIN2* and *STEMIN3*) were activated in leaf cells that underwent reprogramming. In addition, deletion of the three *STEMIN* genes delayed reprogramming after leaf excision, suggesting that these genes redundantly function in the reprogramming of cut leaves. We next examined whether the three *STEMIN* genes also function in stem cell formation in regular protonemal development, formation of chloronema side branch initial cells. We detected promoter activities of *STEMIN1* and its paralogs in chloronema cells undergoing side branch formation (Figure 4). On the other hand, the activities were not detected in chloronema apical stem cells. In addition, the frequency of side branch formation in the triple deletion mutant was significantly lower than that in the wild type. These results suggest that STEMIN1 and its paralogs participate in the formation of chloronema side branch initial cells, but not in the maintenance of chloronema apical stem cells. To understand the role of STEMIN1 in reprogramming, we investigate STEMIN1-direct target genes identified by RNA-seq and ChIP-seq analyses. Masaki Ishikawa and Mio Morishita are this study's main researchers.

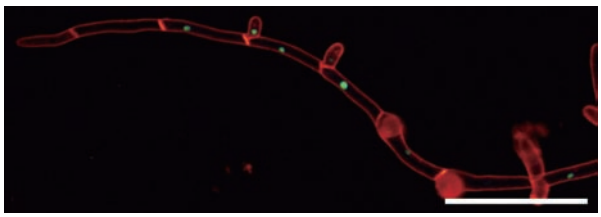


Figure 4. Promoter activity of the STEMIN1 gene in protonemata. Green and red represent the promoter activity and plasma membrane stained with FM4-64, respectively. Scale bar, 100 μm.

VI. Evolution of Regeneration: Stem cells laterally inhibit surrounding cells

Singly isolated leaf cells are reprogramed into stem cells in *P. patens*. However, only one cell of two longitudinally isolated adjacent cells becomes a stem cell and the other appears to be laterally inhibited by the cell to be a future stem cell. The fourth-year graduate student Liechi Zhang is investigating the factors involved in the lateral inhibition.

VII. Evolution of Regeneration: Other pathways

Nan Gu, a fifth-year joint graduate student between Huazon Agricultural University and NIBB is interested in DNA damage and reprogramming, and is working with the mechanisms connecting DNA damage and reprogramming of differentiated cells to stem cells.

We found that INHIBITOR OF GROWTH (ING) proteins are involved in the stem cell formation of cut leaves. The ING proteins are known to regulate an apoptosis pathway in animals but plants do not have the corresponding pathway. Akihiro Imai, a former postdoc in this division and now an Assistant Professor in Hiroshima Institute of Technology is investigating the molecular function of ING as a collaboration work.

VIII. Molecular mechanisms of Plant Movement using *Mimosa pudica*

The sensitive plant *Mimosa pudica* has long attracted the interest of researchers due to its spectacular leaf movements in response to touch or other external stimuli. Although various aspects of the seismonastic movement have been elucidated by physiological, cytological or biochemical approaches, the lack of genetic tools hampered the investigation of molecular mechanisms involved in these processes. To overcome this obstacle, we sequenced and analyzed the genome in a collaborative project with Dr. Chao-Li Huang in National Cheng Kung University. Furthermore, we developed an efficient genetic transformation method for *M. pudica* (Mano *et al*., 2014) and established a CRISPR/Cas9-mediated gene knock-out system. This year we isolated several candidate genes that may play roles in the seismonastic movement by comparing gene expression profiles between motor organs (pulvini) and non-motor organs and between extensor and flexor halves of pulvini. Functional analyses of these genes with the CRISPR/Cas9 system are in progress.

In addition to the reverse genetic analyses, we have challenged whole-organ 3D imaging of tertiary pulvini to comprehensively understand the volume and shape changes of individual cells during movements. To this end, we extensively optimized fixation, staining and tissue clearing methods and succeeded in obtaining images that covered the entire region of a tertiary pulvinus with sufficient resolution. We have also been developing computational methods to automatically extract and analyze the volumes and shapes of thousands of cells in a pulvinus. This study was conducted mainly by Hiroaki Mano.

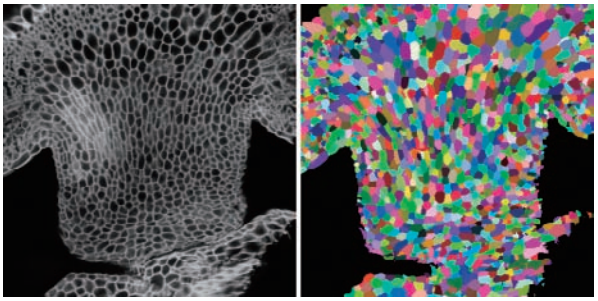


Figure 5. Quantitative 3D image analysis of a tertiary pulvinus of *Mimosa pudica* at the whole organ level.
Left: Cell walls stained by a newly developed method.
Right: Automatic segmentation of the individual cells

IX. Evolution of plant development

To investigate evolution of novel complex traits, the following studies are ongoing with graduate students: The fifth-year graduate student Chiharu Kamida studies genes involved in movable tentacle development in the sandew *Drosera spatulata*. The fourth-year graduate student Shizuka Koshimizu is interested in the evolution of floral homeotic genes and investigates the function of MADS-box genes in the non-flowering plant *Physcomitrella patens*. The pseudanthium is a flower-like inflorescence, the molecular mechanisms of the development of which are unknown. The fifth-year graduate student Tomomi Sugaya succeeded in transferring the *FT* gene from *Arabidopsis thaliana* into the pseudanthium *Houttuynia cordata*. Furthermore, introduction of the *FT* gene successfully induced flowers.

Publication List:

〔Original papers〕

- Tong, W., Imai, A., Tabata, R., Shigenobu, S., Yamaguchi, K., Yamada, M., Hasebe, M., Sawa, S., Motose, H., and Takahashi, T. (2016). Polyamine resistance is increased by mutations in a nitrate transporter gene NRT1.3 (AtNPF6.4) in *Arabidopsis thaliana*. Front. Plant Sci. *7*, 1–22.
- Pereman, I., Mosquna, A., Katz, A., Wiedemann, G., Lang, D., Decker, E.L., Tamada, Y., Ishikawa, T., Nishiyama, T., Hasebe, M., et al. (2016). The Polycomb group protein CLF emerges as a specific tri-methylase of H3K27 regulating gene expression and development in *Physcomitrella patens*. Biochim. Biophys. Acta *1859*, 860–870.
- Plavskin, Y., Nagashima, A., Perroud, P.-F., Hasebe, M., Quatrano, R.S., Atwal, G.S., and Timmermans, M.C.P. (2016). Ancient trans-acting siRNAs confer robustness and sensitivity onto the auxin response. Dev. Cell *36*, 276-289.
- Nagata, T., Hasebe, M., Toriba, T., Taneda, H., and Crane, P.R. (2016). Sex conversion in *Ginkgo biloba* (Ginkgoaceae). J. Jap. Bot. *91 Suppl*., 120-127.

〔Review article〕

- Hasebe, M. (2016). Starting bell for embryos. Nat. Plants *2*, 1-2.

DIVISION OF SYMBIOTIC SYSTEMS

Professor
KAWAGUCHI, Masayoshi

Associate Professor
SOYANO, Takashi

Assistant Professor:	*TAKEDA, Naoya*
Technical Staff:	*FUKADA-TANAKA, Sachiko*
Visiting Scientist	*NAKAGAWA, Tomomi*
Postdoctoral Fellow:	*FUJITA, Hironori*
	KOBAYASHI, Yuuki
	NAGAE, Miwa
	MAEDA, Taro
	KAMEOKA, Hiromu
	SHIMIZU-MITAO, Yasushi
	TOKUMOTO, Yuji
	OKUMA, Nao
SOKENDAI Graduate Student:	*TSUZUKI, Shusaku*
	NISHIDA, Hanna
	FUKUHARA, Mai
	LIU, Meng
Technical Assistant:	*SUZAKI, Momoyo*
	OGAWA, Yuko
	YOSHINORI, Yumi
	TOKAIRIN, Asami
	ODA, Akiko
Secretary:	*OKUBO, Masayo*

Rhizobium–legume symbiosis is one of the most successful mutually beneficial interactions on earth. In this symbiosis, soil bacteria called rhizobia supply the host legumes with ammonia produced through bacterial nitrogen fixation. In return, host plants provide the rhizobia with their photosynthetic products. To accomplish this biotic interaction, leguminous plants develop nodules on their roots. On the other hand, more than 80% of land plant families have symbiotic relationships with arbuscular mycorrhizal (AM) fungi. Despite marked differences between the fungal and bacterial symbioses, common genes are required for both interactions. Using a model legume *Lotus japonicus*, we are trying to unveil the molecular mechanisms of both symbiotic systems.

I. Root nodule symbiosis

1-1 A third CLE peptide systemically controls nodulation in *L. japonicus*.

In root nodule symbiosis, a mutual relationship between leguminous plants and nitrogen-fixing rhizobia, the mechanism for the autoregulation of nodulation (AON) plays a key role in preventing the production of an excess number of nodules. AON is based on long-distance cell-to-cell communication between roots and shoots. Among the 39 *CLAVATA3/ESR-related* (*CLE*) peptide genes identified from *Lotus japonicus*, the expression of *CLE-ROOT SIGNAL 1* (*CLE-RS1*) and *-RS2* is induced immediately in response to rhizobial inoculation after direct activation by an RWP-RK type transcription factor NODULE INCEPTION (NIN). CLE-RS1 and -RS2 peptides act as putative root-derived signals that transmit signals inhibiting further nodule development through interaction with a shoot-acting receptor-like kinase HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1). A loss-of function mutation in the *HAR1* gene significantly increases nodule numbers. Hence, the CLE-RS1/2–HAR1 pathway is hypothesized to play a pivotal role in the negative regulation of nodulation in AON. KLAVIER (KLV), another shoot-acting LRR-RLK, seems to be involved in CLE-RS1/2-mediated negative regulation of nodulation. Recently cytokinin production was reported to be induced in the shoot by the downstream part of the CLE-RS1/2–HAR1 signaling pathway. In addition, shoot-applied cytokinin is able to move to roots and inhibit nodulation. These results suggest that shoot-derived cytokinin may be a shoot-derived inhibitor (SDI) candidate. There might be a proteasome-mediated degradation process for an unidentified protein in the most downstream part of AON in roots because the negative effect of shoot-applied cytokinin is masked by a mutation in the F-box protein TOO MUCH LOVE (TML). Although our knowledge of AON has been furthered, identification of additional components of AON will be undoubtedly essential for a deeper understanding of the mechanism.

In order to identify new *LjCLE* genes, we referred to a new database that has a reference sequence data set containing the *L. japonicus* genome assembly Lj2.5 and the unique de novo assembled contigs derived from *L. japonicus*. A BLAST search using the amino acid sequence of a CLE domain from CLE-RS1 as a query enabled us to identify five new CLE peptides. Among the CLE peptides identified, *CLE-RS3* and *LjCLE40* expression was induced in inoculated roots. A hairy root transformation study showed that constitutive expression of *CLE-RS3* in the roots significantly reduced nodule number not only in transformed but also in untransformed roots. On the other hand, the CLR-RS3-mediated suppression of nodulation activity was masked in the *har1* and *tml* plants. These results suggest that CLE-RS3 is a new component of AON in *L. japonicus* that may act as a potential root-derived signal.

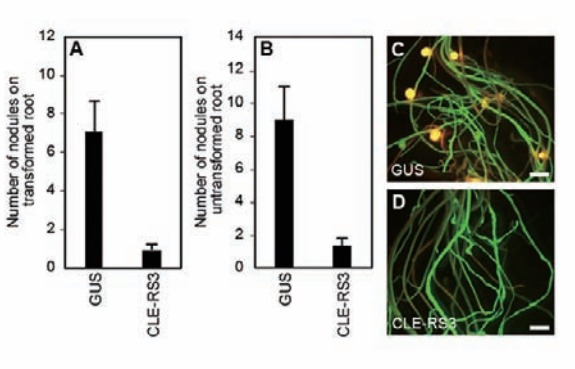


Figure 1. Number of nodules formed on transformed (A) and untransformed (B) roots of the wild-type plants that have transgenic hairy roots constitutively expressing *GUS* or *CLE-RS3* (n = 14–20 plants). The nodulation phenotype of the plants that have transgenic hairy roots constitutively expressing *GUS* (C) or *CLE-RS3* (D) at 21 days after inoculation. Transgenic roots were identified by GFP fluorescence. The *Mesorhizobium loti* strain that constitutively expresses *DsRED* was used in these experiments. Error bars indicate SE. Scale bars 2 mm.

1-2 Thiamine biosynthesis is required for nodule development.

Thiamine (vitamin B1) is an essential nutrient to produce energy. Thiamine is synthesized through a multiple-step pathway and functions in the form of a thiamine pyrophosphate. Thiamine is assembled from pyrimidine and thiazole moieties. In *L. japonicus*, THI1 and THI2 (a THI1 paralog) catalyze the biosynthesis of the thiazole moiety, and THIC catalyzes the biosynthesis of the pyrimidine moiety.

The phenotypes of thiamine-deficient mutants of *L. japonicus* are summarized in Figure 2. *THIC* is expressed in all tissues and is a single copy gene in *L. japonicus*. The *thiC* mutant showed chlorosis in the leaves (Figure 2A), which is a typical and lethal phenotype observed in the thiamine-deficient plants. *THIC* expression was induced in nodules, and the nodule number also showed a reduction in the *thiC* mutant. However, it is not clear that the nodulation defect in the *thiC* mutant is caused by the loss of *THIC* function, because the chlorosis resulted in severe growth defects which also caused a decrease of nodule formation. Therefore, we also analyzed *THI1* function in nodulation. *THI1* is highly expressed in roots, nodules, and seeds, whereas *THI2* is expressed mainly in shoots. The *thi1* mutant did not have chlorosis in the leaves and showed no significant growth defects, although the knockdown plants of *THI2* gene displayed chlorosis and growth defects. The *thi1* mutant showed reduced nodule and seed size (Figure 2B, C), and the phenotypes were suppressed by exogenous thiamine treatment. The analyses indicated that thiamine affects the early stage of nodule development. These results demonstrated that THI1 is involved in both nodule development in roots and seed maturation in shoots, excluding the effects of chlorosis and growth defects.

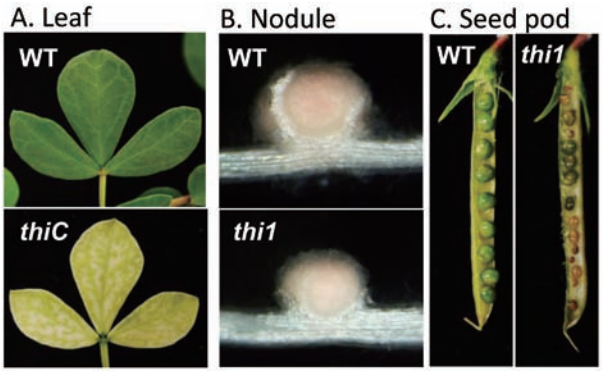


Figure 2. Thiamine-deficient phenotypes in *thiC* and *thi1* mutants of *L. japonicus*. The *thiC* mutant showed chlorosis in leaves (A). The nodule size decreased in the *thi1* mutant (B). The *thi1* mutant also showed abnormal phenotypes in seed formation (C).

On the other hand, we could not observe obvious AM colonization phenotypes in the *thi1* mutant or thiamine-treated plants. However, it has been reported that the AM fungus *Rhizophagus irregularis* lacks thiamine biosynthesis genes. Thiamine is essential for living organisms, therefore, AM fungus should require a thiamine supply from the host plant. We think that further analysis is required to reveal thiamine function and effect on AM in both the host plant and AM fungus.

II. Improvement of referential AM fungus genome

Arbuscular mycorrhiza (AM) is a mutualistic plant-fungus interaction that confers great advantages to growth and survival on the land. However, the molecular biological mechanisms governing the symbiotic relationships remain largely unknown. The fragmented genome data of AM fungi (AMF) had been one of the barriers for the molecular biological study of AM. Although the genome of a model strain of AMF, *R. irregularis* DAOM-181602, has been sequenced in multiple studies, these genomic data were made up of over 28,000 short sequences (N50 = 4-16kbp). Thus, previous data was difficult to use for comparative genomics with other fungal species.

To facilitate the molecular biology of AMF, we improved *R. irregularis* whole-genome data using PacBio-based *de novo* sequencing. As a result, the total size of our 210 contigs reached 97.2 % (149.75 Mbp) of the predicted genome size (154 Mb), and its N50 length elongated to 2.2 Mbp. Compared to previous analyses, the genome completeness in total assembly size increased 6-39 points, the number of assemblies decreased by135-144 fold, and N50 length became about 140-551 times longer (Figure 3). This improvement of the statistics validated the availability of the PacBio sequencing to the repeat-rich genome.

From the new assemblies, we constructed 37,711 protein-coding genes. This gene model comprised 94% of the fungal core conservative genes, suggesting high genetic completeness of our gene model set. However, our genomic data did not contain some of the key genes for the typically present metabolic pathway in autotrophic fungi (e.g., Thiamine synthesis). This is credible evidence of gene loss in *R. irregularis* genomes, and supports the previous opinion that AMF are unable to produce those essential nutrients. AMF may obtain the nutrients from the host plant. Overall, we succeed in providing a high-quality reference genome data for the molecular understanding of AM.

III. Pattern density control in self-organized pattern formation

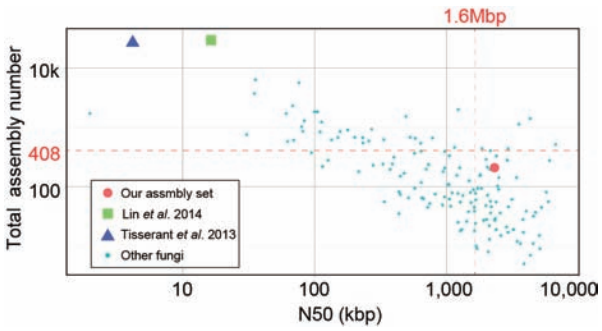


Figure 3. Assembly statistics of *R. irregularis* and other fungus genomic data. The correspondence between the symbols and the assembly sets are presented in the boxed legend. The statistics (total assembly size and N50 length) from *R. irregularis* were presented with larger symbols. The other 153 fungal genomes from the RefSeq database are presented with blue dots. The horizontal and vertical red lines represent averages of total assembly numbers and N50 length of the 153 fungal genomes.

Many self-organized patterns have been explained by the concept of the Turing mechanism, in which interactions between diffusible molecules initiate spatial instability to translate into stable patterns. Whether or not spatial instability is induced can be determined by conventional linear stability analysis. In contrast, resulting spatial patterns produced by such instability depend on nonlinear effects of the model dynamics and are difficult to be predicted without numerical simulations.

In two-dimensional space, patterns generated by the Turing system are divided into three types: spot patterns, stripe patterns, and reverse spot patterns (Figure 4, upper panels). It is reported that these pattern types are associated with the relative position of the equilibrium between lower and upper constraints in the activator–inhibitor system, one of the best-known Turing systems. That is, spot, stripe, and reverse spot patterns are formed when the equilibrium is closer to the lower limit, around the middle of the two limits, and closer to upper limit, respectively.

We here report that pattern density, proportion of area with high concentrations of the activator molecule, is strongly correlated with R_{eq}, the relative position of the equilibrium between the upper and lower constraints, in linear dynamics of the activator–inhibitor system (Figure 4). Furthermore, we demonstrate that this finding can be successfully applied to the well-known phenomenon of animal skin color pattern formation and to the patterning of stomatal lineage. This relationship between equilibrium position and pattern density is also observed in various nonlinear dynamics. Accordingly, this finding could be widely applicable to self-organized patterns and would be a powerful and reliable tool for elucidating the underlying mechanism of self-organized pattern formations in biological systems.

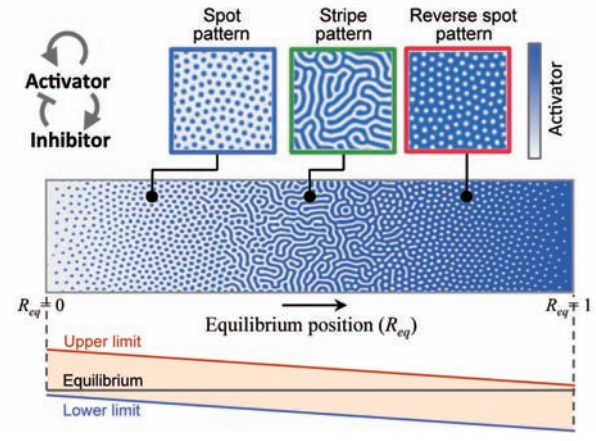


Figure 4. Pattern density control by activator-inhibitor dynamics with upper and lower constraints in two-dimensional space. Pattern density (proportion of area with high activator concentrations) increases as equilibrium becomes apart from lower limit and close to upper limit (i.e. R_{eq} increases). Insets show activator distributions that correspond to spot pattern (R_{eq} = 0.2), tripe pattern (R_{eq} = 0.5), and reverse spot pattern (R_{eq} = 0.8).

Publication List:

〔Original papers〕

- Kameoka, H., Dun, E.A., Lopez-Obando, M., Brewer P.B., de Saint Germain, A., Rameau, C., Beveridge, C.A., and Kyozuka, J. (2016). Phloem transport of the receptor DWARF14 protein is required for full function of strigolactones. Plant Physiol. *172*, 1844–1852.
- Kikuchi, Y., Hijikata, N., Ohtomo, R., Handa, Y., Kawaguchi, M., Saito, K., Masuta, C., and Ezawa, T. (2016). Aquaporin-mediated long-distance polyphosphate translocation directed towards the host in arbuscular mycorrhizal symbiosis: application of virus induced gene silencing. New Phytol. *211*, 1202-1208.
- Malolepszy, A., Mun, T., Sandal, N., Gupta, V., Dubin, M., Urbański, D., Shah, N., Bachmann, A., Fukai, E., Hirakawa, H., Tabata, S., Nadzieja, M., Markmann, K., Su, J., Umehara, Y., Soyano, T., Miyahara, A., Satoh, S., Hayashi, M., Stougaard, J., and Andersen, S. (2016). The LORE1 insertion mutant resource. Plant J. *88*, 306-317.
- Miyata, K., Hayafune, M., Kobae, Y., Kaku, H., Nishizawa, Y., Masuda, Y., Shibuya, N., and Nakagawa, T. (2016). Evaluation of the role of the LysM receptor-like kinase, OsNFR5/OsRLK2 for AM symbiosis in rice. Plant Cell Physiol. *57*, 2283-2290.
- Miyata, K., and Nakagawa, T. (2016). The *Lotus* intrinsic ethylene receptor regulates both symbiotic and non-symbiotic responses. Plant Biotech. *33*, 27-32.
- Nagae, M., Parniske, M., Kawaguchi, M., and Takeda, N. (2016). The thiamine biosynthesis gene *THI1* promotes nodule growth and seed maturation. Plant Physiol. *172*, 2033-2043.
- Nagae, M., Parniske, M., Kawaguchi, M., and Takeda, N. (2016). The relationship between thiamine and two symbioses: root nodule symbiosis and arbuscular mycorrhiza. Plant Signal. Behav. *11*, e1265723.
- Nishida, H., Handa, Y., Tanaka, S., Suzaki, T., and Kawaguchi, M. (2016). Expression of the CLE-RS3 gene suppresses root nodulation in *Lotus japonicus*. J. Plant Res. *129*, 909-919.
- Sakurai, K., Tomiyama, K., Kawakami, Y., Ochiai, N., Yabe, S., Nakagawa, T., and Asakawa, Y. (2016). Volatile components emitted from the liverwort *Marchantia paleacea* subsp. *diptera*. Nat. Prod. Commun. *11*, 263-264.
- Tokumoto, Y., and Nakagawa, M. (2016). Climate-induced abortion and predation: reproductive success of the pioneer shrub *Dillenia suffruticosa* in Malaysian Borneo. J. Trop. Ecol. *32*, 50-62.
- Tokumoto, Y., Kajiura, H., Takeno, S., Harada, Y., Suzuki, N., Hosaka, T., Gyokusen, K., and Nakazawa, Y. (2016). Induction of tetraploid hardy rubber tree, *Eucommia ulmoides*, and phenotypic differences from diploid. Plant Biotechnol. *33*, 51-57.
- Tsuzuki, S., Handa, Y., Takeda, N., and Kawaguchi, M. (2016). Strigolactone-induced putative secreted protein 1 is required for theestablishment of symbiosis by the arbuscular mycorrhizal fungus Rhizophagusirregulars. Mol. Plant Microbe Interact. *29*, 277-286.

〔Original paper (E-publication ahead of print)〕

- Yano, K., Aoki, S., Liu, M., Umehara, Y., Suganuma, N., Iwasaki, W., Sato, S., Soyano, T., Kouchi, H., and Kawaguchi, M. Function and evolution of a *Lotus japonicus* AP2/ERF family transcription factor that is required for development of infection threads. DNA Res. 2016 Dec 27.

DIVISION OF EVOLUTIONARY DEVELOPMENTAL BIOLOGY

Professor
NIIMI, Teruyuki

Assistant Professor:	*OHDE, Takahiro*
	ANDO, Toshiya
Technical Staff:	*MIZUTANI, Takeshi*
NIBB Research Fellow:	*MORITA, Shinichi*
Visiting Graduate Student:	*MASE, Mutsuki*
	YATOMI, Johichiro
	YUZAKI, Karen
	KONISHI, Yusuke
Technical Assistant:	*KAWAGUCHI, Haruka*
	MORITA, Junko
Secretary:	*SAITO, Eiko*

The Division of Evolutionary Developmental Biology was started in June 2015. We focus on the evolutionary novelties acquired by insects through evolution, in order to elucidate the molecular and evolutionary mechanisms leading to the large variety of traits that they display. From this wealth of exciting traits, our lab currently focuses on promoting research into (1) the origin and diversification of insect wings, (2) wing color patterns and mimicry of ladybird beetles, and (3) acquisition and diversification of beetle horns.

I. Origin and diversification of insect wings

Of the various flying animals on the earth, insects have evolved a unique flight organ. Despite over two centuries of debate, the evolutionary origin of the insect wing is still an enigma. We try to approach this issue using evo-devo methods. In *Drosophila melanogaster*, the wing master gene *vestigial* (*vg*) and its interaction partner *scalloped* (*sd*) play pivotal roles in the formation of wing field identity. For this reason, these genes are ideal candidates for investigating wing origin and evolution.

One way to identify the structure from which insect wings first evolved is to explore the function of "wing" genes in ancestral wingless (apterygote) species. We chose the firebrat, *Thermobia domestica,* as a model (Figure 1A). *T. domestica* belongs to Thysanura, phylogenetically the closest extant relative of winged (pterygote) insects, making it ideal for elucidating wing origin. We cloned *vg* and *sd* orthologs from *T. domestica* (*Td-vg* and *Td-sd*). To examine the functions of these genes, we developed RNA interference (RNAi) based methods for *T. domestica*. We are currently testing for functional effects of altered transcription of each of these wing genes in the ancestrally wingless firebrats. In addition, we are performing comparative analyses of the function of these same genes in "primitively winged" (hemimetabolous) insects, to obtain additional clues relevant to understanding the origin and evolution of insect wings.

Interestingly, our previous work showed that *vg* expressing epidermal tissue forms lateral outgrowths in non-winged segments in the mealworm beetle (Ohde *et al*., 2013). From these facts, we hypothesize that ancestral lateral body wall outgrowths evolved into functional wings. To test this hypothesis, we are now comparing the role of *vg* and other "wing genes" between wings and lateral outgrowths in a basal winged insect, *Gryllus bimaculatus,* and non-winged insect, *Thermobia domestica* (Figure 1).

II. Wing color patterns and mimicry of ladybird beetles

Insect wing color patterns demonstrate a tremendous range of diversity and have evolved to fulfill various ecologically important functions such as intraspecific sexual signaling, mimesis, mimicry, and warning against predators. The molecular mechanisms responsible for generating such patterns, however, remain unknown for most species. To investigate the developmental mechanisms of color pattern formation, we chose the multicolored Asian ladybird beetle, *Harmonia axyridis*, which has conspicuous and variable wing color patterns consisting of black and red pigments (Figure 2A). Vivid wing color patterns of ladybirds function as a warning signal to predators that they are distasteful, and ladybird beetles are mimicked by various insect species. Mimicry provides an exciting opportunity to study how independent lineages of insects converge on similar color patterns. For exploring color pattern formation in a mimic, we use the leaf beetle, *Argopistes coccinelliformis*, which has color patterns similar to *Harmonia*, and which is thought to be a Batesian mimic of ladybird beetles (Figure 2B). To elucidate the molecular mechanisms underlying these wing color patterns, we have established a technique for germline transformation using a *piggyBac* vector and RNAi in the ladybirds.

Recently, genome-editing technologies using TALEN and CRISPR/Cas9 provide rapid and potent genetic analysis systems including not only gene knockout but also gene knock-in. To establish TALEN-mediated genome editing in *Harmonia*, we used a transgenic ladybird strain that has single copies of exogenous *EGFP* and *N-acetyl transferase* (NAT, anti-melanin synthesis enzyme) genes. Messenger

Figure 1. The firebrat, *Thermobia domestica* (A). the two-spotted cricket, *Gryllus bimaculatus* (B).

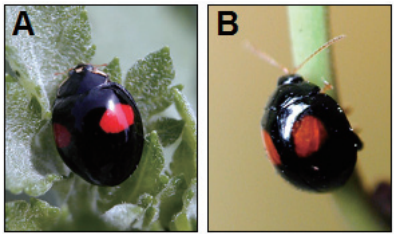


Figure 2. The ladybird beetle, *Harmonia axyridis* (A) and the leaf beetle, *Argopistes coccinelliformis* (B).

RNAs of TALENs targeting only the EGFP transgene were microinjected into the fertilized eggs. As a result, 20.8% of G0 founders produced EGFP-negative and NAT-positive knockout progenies. This efficiency was higher than that of germline transformation by *piggyBac* vectors (about 5%). We are continuing to develop both CRISPR/Cas9 and *piggyBac* methods in our lab and will use both approaches to perturb expression of candidate genes putatively involved in wing color pattern formation.

Based on the knowledge obtained from *H. axyridis*, we are trying to understand how the similar wing-color patterns of model and mimic are generated – for example, do they use conserved or divergent mechanisms?

III. Acquisition and diversification of beetle horns

Insects show a tremendous range of diversity in "horns", rigid body outgrowths that function as weapons. Horns are exciting for evo-devo studies because they have arisen multiple times *de novo,* as evolutionary "novelties". However, the molecular mechanisms involved in sexually dimorphic horn formation are still poorly understood. To investigate the developmental mechanisms of horn formation, we focus on the Japanese rhinoceros beetle, *Trypoxylus dichotomus* (Coleoptera), which exhibits remarkable sexual dimorphisms in head and thoracic horns (Figure 3A). The male-specific horns of *T. dichotomus* are one of the best models to study how an extreme, sex-specific morphology is formed. We have developed a technique for larval RNAi in *T. dichotomus,* permitting us to rigorously and systematically test the functional roles of a large suite of candidate developmental genes, revealing for the first time the molecular mechanisms responsible for growth of male rhino beetle horns. In addition, we are employing a high throughput approach. To identify novel genes involved in the sexually dimorphic horn development in *T. dichotomus*, mRNA of the developing horn discs has been assessed by deep-sequencing transcriptome analysis.

To understand how, molecularly, beetle horns have diversified, we are extending our analyses to include additional beetle species with different types of exaggerated horns, including rhinoceros beetles with diverse horn structures as well as horned beetles in other phylogenetic groups (Figure 3).

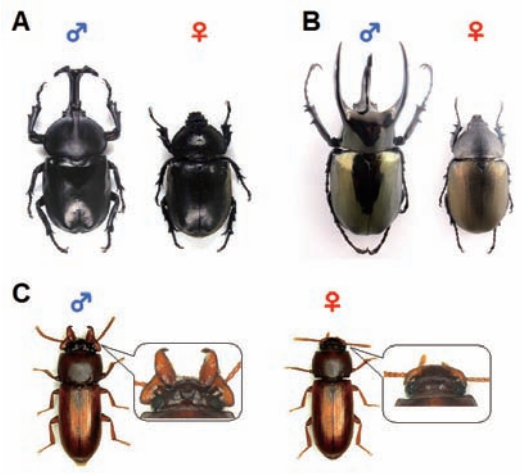


Figure 3. The Japanese rhinoceros beetle, *Trypoxylus dichotomus* (A), the Atlas beetle, *Chalcosoma atlas* (B) and the broad-horned flour beetle, *Gnatocerus cornutus* (C). Adult male (Left) and female (Right).

Previously, we have shown that the sex-determination gene *doublesex* (*dsx*) plays crucial roles regulating horn development in *T. dichotomus* (Scarabaeoidea, Scarabaeidae) (Ito *et al*., 2013). The function of *dsx* on weapon traits in Scarabaeoidea is conserved among beetles tested so far. Although sex-specific weapon traits have evolved independently in various Coleopteran groups, developmental mechanisms of sex-specific expression have not been studied outside of the Scarabaeoidea. Therefore, we focused on the sexually dimorphic broad-horned flour beetle *Gnatocerus cornutus* (Tenebrioidea, Tenebiriodae). In this beetle, only males have well-developed mandibles and genae, and a pair of short horns on the head (Figure 3C). We investigated the function of *dsx* in this beetle using larval RNAi. Our results show that sex-specific *G. cornutus dsx* isoforms have an antagonistic function for all sexually dimorphic head structures. Combining these results with previous studies in other sexually-dimorphic weaponed beetles in the Scarabaeoidea, indicated that sex-specific regulation of weapon development via differential Dsx isoforms is a common mechanism shared by sexually-dimorphic beetles, even though their specific weapon traits have evolved independently.

Publication List:

〔Original papers〕

- Gotoh, H., Ishiguro, M., Nishikawa, H., Morita, S., Okada, K., Miyatake, T., Yaginuma T., and Niimi, T. (2016). Molecular cloning and functional characterization of the sex-determination gene *doublesex* in the sexually dimorphic broad-horned beetle *Gnatocerus cornutus* (Coleoptera, Tenebrionidae). Sci. Rep. *6*, 29337.
- Gotoh, H., Zinna, R.A., Warren, I., DeNieu, M., Niimi, T., Dworkin, I., Emlen, D.J., Miura, T., and Lavine, L.C. (2016). Identification and functional analyses of sex determination genes in the sexually dimorphic stag beetle *Cyclommatus metallifer*. BMC Genom. *17*, 250.
- Ozawa, T., Mizuhara, T., Arata, M., Shimada, M., Niimi, T., Okada, K., Okada, Y., and Ohta, K. (2016). Histone deacetylases control module-specific phenotypic plasticity in beetle weapons. Proc. Natl. Acad. Sci. USA *113*, 15042-15047.
- Zinna, R., Gotoh, H., Brent, C.S., Dolezal, A., Kraus, A., Niimi, T., Emlen, D., and Lavine, L.C. (2016). Endocrine control of exaggerated trait growth in rhinoceros beetles. Integr. Comp. Biol. *56*, 247-259.

LABORATORY OF BIORESOURCES

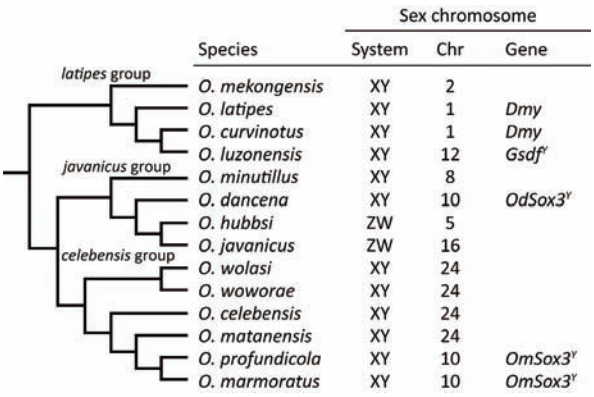
Specially Appointed Professor
NARUSE, Kiyoshi

Assistant Professor:	*TAKEHANA, Yusuke*
Postdoctoral Fellow:	*CHISADA, Shinichi*
	SASADO, Takao
	YOKOI, Saori
Research Fellow:	*KANEKO, Hiroyo*
Visiting Scientist:	*KIMURA, Tetsuaki*
Technical Assistant:	*AJIOKA, Rie*
	HARA, Ikuyo
	KOIKE, Chieko
	KOIKE, Yukari
	TAKAKI, Chikako
	TESHIMA, Yuuko
	TORII, Naoko
	YAMAZAKI, Toko
	YANOGAWA, Azusa
Secretary:	*SUZUKI, Tokiko*

Medaka is a small egg-laying "secondary" fresh water fish found in brooks and rice paddies in Eastern Asia. This species has a long history as an experimental animal, especially in Japan. Our laboratory has conducted studies on evolution of the sex determination system using medaka and relatives, identification of the causal gene of mutants for PGC migration and pigment cell development, development of human disease models, and the genetic and neural basis of sexual behavior in medaka. In addition to these activities, our laboratory is stepping forward to lead the National BioResource Project Medaka (NBRP Medaka).

I. Evolution of the sex chromosome and sex-determining genes in *Oryzias* fish

Sex chromosomes harbor a primary sex-determining signal that triggers sexual development of the organism. In mammals, *Sry* is the dominant male-determining gene located on the Y chromosome, and has evolved from the neural gene *Sox3* on the X chromosome probably through a regulatory mutation. However, independent evolution of sex chromosomes is widespread in non-mammalian vertebrates, suggesting that sex determination mechanisms are regulated by different genes and have evolved rapidly. Medaka fishes in the genus *Oryzias* have different sex chromosomes with different systems (XY and ZW), providing ideal conditions for investigating the mechanisms that lead to the rapid turnover of sex chromosomes (Figure 1). So far, different sex-determining genes, *Dmy* and *Gsdf*^{Y}, have been isolated from the *Oryzias* species, demonstrating that turnover of sex chromosomes is associated with the substitution of master sex-determining genes. Recently, we identified *Sox3* as a novel sex-determining gene on the XY sex chromosomes in the marine medaka *Oryzias dancena/melastgma* by positional cloning. Sex reversed phenotypes in transgenic fish and loss-of-function mutants of the Y chromosomal *Sox3* allele all point to its critical role in sex determination, suggesting that the neo-Y chromosome of *O. dancena* arose by co-option of *Sox3*. Furthermore, we found the *Sox3* gene also on the XY sex chromosomes in distantly related *Oryzias* species, *O. marmoratus* and *O. profundicola*. Fine mapping and association analysis identified the Y chromosome-specific 430-bp insertion at the *Sox3* locus, which appeared to be involved in its male determination function. The *Sox3*-dependent sex determination system in *Oryzias* species is polyphyletic, and the Y-specific insertion has not been found in *O. dancena*, suggesting that *Sox3* has evolved as the sex-determining gene independently in different lineages of *Oryzias*. These results suggest that *Sox3* might have acquired the novel male-determining function repeatedly and independently during vertebrate evolution.



Species	Sex chromosome System	Chr	Gene
O. mekongensis	XY	2	
O. latipes	XY	1	*Dmy*
O. curvinotus	XY	1	*Dmy*
O. luzonensis	XY	12	*Gsdf*^{Y}
O. minutillus	XY	8	
O. dancena	XY	10	*OdSox3*^{Y}
O. hubbsi	ZW	5	
O. javanicus	ZW	16	
O. wolasi	XY	24	
O. woworae	XY	24	
O. celebensis	XY	24	
O. matanensis	XY	24	
O. profundicola	XY	10	*OmSox3*^{Y}
O. marmoratus	XY	10	*OmSox3*^{Y}

Figure 1. Phylogenetic relationships and sex determination mechanisms in *Oryzias* fishes.

II. The study of type 2 diabetes using leptin receptor knockout medaka

Leptin in mammals is a peptide hormone secreted by adipose tissue. It has been shown to play a key role in the maintenance of energy homeostasis through the regulation of food intake and a range of physiological functions. Mice with a deficiency of leptin or its receptor exhibit hyperphagia (an increase in food intake). The hyperphagia causes obesity leading to type 2 diabetes-like symptoms, which is consistent with Caucasian patients. Leptin has also been isolated from fish, including medaka, however, the amino acid sequence is poorly conserved between fish and mammals (11-30%), and fish leptins are expressed mainly in the livers. To clarify the function of leptin on fish, we generated leptin receptor knockout (LepRKO) medaka by the TILLING method. The phenotypic analyses allowed us to reveal an appetite suppressive function of leptin signaling on medaka as well as mammals, and to find new value in medaka as a novel animal model for studying type 2 diabetes. As for appetite suppressive functions; LepRKO medaka showed high expression of the mRNA of NPY (3.5-fold) and AgRP (6-fold), which are known to be orexigenic peptides, and an increase in food intake (1.7-fold). Next, as for glucose metabolism; adult mutants showed signs of diabetes, such as fasting hyperglycemia and impaired insulin secretion, which is a late-onset disorder caused by excessive feeding during post-juvenile stages. Furthermore, they showed hyperglycemia even with the same fat level in the blood, muscle, and liver as WT medaka. The symptom is consistent with those of Asian patients, not but Caucasian patients and mice with leptin signaling deficiencies. Now, we are investigating the

gene expression associated with dysfunction of pancreatic tissues under various feeding conditions. This will allow us to identify the factors of diabetes that are sensitive to food intake, regardless of obesity.

III. Adaptive significance of persistent mate-guarding behavior in medaka

Males of various animals exhibit mate-guarding behavior to prevent rival males from mating with the female. Most of them, however, exhibit this behavior during only the mating period, because persistent mate-guarding is thought to have a high energy cost, which would reduce male survival rate. Previously, we reported that medaka males exhibit mate-guarding irrespective of the mating period (Yokoi et al., 2015) and it remains unknown whether there is some benefit of the persistent mate-guarding, such as enhancement of male reproductive success, in medaka fish. In addition, medaka females tend to choose visually-familiarized males as their mating partner and the adaptive significance of this female mating preference is totally unknown. Recently, we found that mate-guarding led to familiarization with the female while at the same time blocking the female's visual familiarization with other males in medaka fish. We put three fish (female, male, male) separately in a transparent three-chamber tank, which allowed the male in the center (near male) to maintain closer proximity to the female than the other male (far male). Placement of the wild-type male in the center blocked visual familiarization of the far male by the female via mate-guarding. In contrast, placement of an arginine-vasotocin receptor mutant male, which exhibits mate-guarding deficits, in the center, allowing for maintaining close proximity to the female, did not block familiarization of the far male by the female. This finding suggested that persistent mate-guarding allows males to gain familiarity with the female over their rivals, which may enhance female preference for the dominant male (Figure 2). We hope that this study helps us understand the evolutional origin of "pair-bonding".

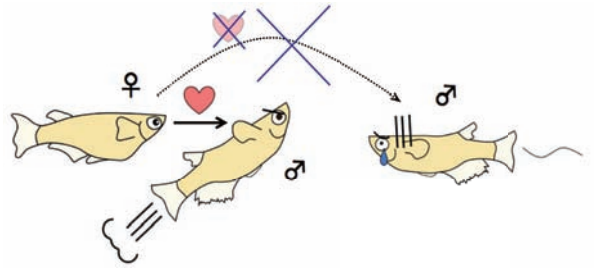


Figure 2. Persistent mate-guarding blocks familiarization between the female and the rival male, which may enhance female preference for the dominant male.

IV. National BioResource Project Medaka (NBRP Medaka) (http://www.shigen.nig.ac.jp/medaka/)

In 2007, NIBB was selected as the core facility of NBRP Medaka. Our laboratory is taking an active part in this project. With the goal of facilitating and enhancing the use of medaka as a model organism, we provide, maintain and collect living resources such as standard strains, inbred strains, and mutants in addition to frozen resources such as EST/cDNA, BAC/ Fosmid clones, and hatching enzymes, as well as integrated information on medaka. We have been providing BAC clones of medaka related species, a library screening system employing a 3D PCR strategy for evolutionary studies, and the TILLING screening system and CRISPR-Cas9 genome editing platform for promoting the reverse genetic approach. NBRP Medaka aims to establish a first rate biological resource with the highest possible levels of accessibility and ease of use.

Publication List:

〔Original papers〕

- Edeline, E., Terao, O., and Naruse, K. (2016). Empirical evidence for competition-driven semelparity in wild medaka. Popul. Ecol. *33*, 246-254.
- Isoe, Y., Konagaya, Y., Yokoi, S., Kubo, T., and Takeuchi, H. (2016). Ontogeny and sexual differences in swimming proximity to conspecifics in response to visual cues in medaka fish. Zool. Sci. *33*, 246-254.
- Kagawa, N., Honda, A., Zenno, A., Omoto, R., Imanaka, S., Takehana, Y., and Naruse, K. (2016). Arginine vasotocin neuronal development and its projection in the adult brain of the medaka. Neurosc. Lett. *613*, 47–53.
- Komine, R., Nishimaki, T., Kimura, T., Oota, H., Naruse, K., Homma, N., and Fukamachi, S. (2016). Transgenic medaka that overexpress growth hormone have a skin color that does not indicate the activation or inhibition of somatolactin-α signal. Gene *584*, 38-46.
- Takehana, Y., Sakai, M., Narita, T., Sato, T., Naruse, K., and Sakaizumi, M. (2016). Origin of the boundary populations in medaka (*Oryzias latipes* species complex). Zool. Sci. *33*, 125-131.
- Takehana, Y., Matsuda, Y., Ikuta, J., Kryukov, A.P., and Sakaizumi, M. (2016). Genetic population structure of the Japanese grass lizard, *Takydromus tachydromoides* (Reptilia: Squamata), inferred from mitochondrial cytochrome *b* variations. Curr. Herpetol. *35*, 22-32.
- Yokoi, S., Ansai, S., Kinoshita, M., Naruse, K., Kamei, Y., Young, L.J., Okuyama, T., and Takeuchi, H. (2016). Mate-guarding behavior enhances male reproductive success via familiarization with mating partners in medaka fish. Front. Zool. *13*, 21.
- Zhang, X., Guan, G., Li, M., Zhu, F., Liu, Q., Naruse, K., Herpin, A., Nagahama, Y., Li, J., and Hong, Y. (2016). Autosomal *gsdf* acts as a male sex initiator in the fish medaka. Sci. Rep. *6*, 19738.

〔Review article〕

- Naruse, K., Chisada, S., Sasado, T., and Takahana, Y. (2016). Medaka as model animal and current atatus of medaka biological resources. Res. & Knowl. *2*, 31-34.

LABORATORY OF MORPHODIVERSITY

Associate Professor
KODAMA, Ryuji

Visiting Scientist: *YOSHIDA, Akihiro*

The aim of this laboratory is to observe the variety of morphogenetic processes that occur in the course of the ontogenesis of multicellular organisms and to analyze the mechanisms of such processes, mainly by morphological methods. The accumulation of such analyses of the embryogenetic processes of related species is expected to provide an insight into the evolution of morphogenetic processes. This laboratory uses the wings of lepidopteran insects as the main target of morphological studies.

I. Wing outline shape formed by cell death

The wings of the lepidopteran insects (butterflies and moths) develop from the wing imaginal disc, which is a hollow sac made of simple epithelium. Due to its simple construction, this tissue is a good material for studying cellular interactions in the course of morphogenesis.

The outline shape of the adult wing is often different from that of the pupal wing. This difference is brought about by the programmed cell death of the marginal area of the pupal wing. The marginal dying area is called “the degeneration region” and the internal area which develops into the adult wing is called “the differentiation region”.

Cell death in the degeneration region proceeds very rapidly and finishes in a half-day to one-day period in *Pieris rapae* and in several other examined species. It was shown that the dying cells in the degeneration region have characteristics in common with the apoptotic cell death in mammalian cells, such as fragmented and condensed nuclei containing short DNA fragments detected by TUNEL staining. The cells in the degeneration region are actively engulfed by the macrophages in the cavity beneath the wing epithelium. The macrophages seem to be concentrated beneath the degeneration region by the strong adhesion between the basal surfaces of the dorsal and ventral epithelium in the differentiation region, which occurs at the time of prominent cell death and excludes macrophages from the differentiation region. Thus realized concentration of macrophages seems to accelerate the shrinkage of the degeneration region.

A possible physiological role of the cell degeneration at the wing margin is to make space for the growth of the marginal scales. Marginal scales are extremely elongated scales that grow densely on the edge of the wing. These scales are considered to be important in stabilizing the turbulence occurring posterior to the wing. The movements of the marginal scales are closely monitored by sensory scales and bristles growing among them (Yoshida and Emoto, Zool. Sci. *28*, 430-437, 2011).

Trachea and tracheoles are both important in delivering air into the wing and their patterns coincide with that of the boundary of degeneration and differentiation zones at the distal end of the wing. According to previous observations, the pattern formation of wing epithelium is often dependent on tracheal and tracheole patterns. Basic research on the development of tracheal pattern formation is being done by scanning and transmission electron microscopy to describe the exact pathway and time course of the formation of the elaborate pattern of trachea and tracheoles and to establish the cytological and developmental relationship between the formation of the tracheal pattern and the epithelial cell pattern.

II. Wing morphogenesis and the growth of marginal scales in small moths

In the small moths which have very long scales along their wing margins, the cuticle of the pupal wings does not appear to be large enough to house these scales. We examined the developmental process of the pupal wings of three species of the small gelechiid moths and found that, concomitant with the programmed cell deaths at the wing margin, there occurs a shrinkage of the differentiation region which has not been observed in large winged butterflies and moths. The shrinkage concomitant with the cell deaths causes the space between the pupal wing and the cuticle to extend, which appears to contribute to the growth of long marginal scales.

Microscopic observation of the long marginal scales revealed that they have a novel branching morphology and the branches were ubiquitously and densely distributed within the scale array to form a mesh-like architecture similar to a nonwoven fabric. The marginal scales maintain a coherent sheet-like structure during wingbeat. (Yoshida, A., Tejima, S., Sakuma, M., Sakamaki, Y., and Kodama, R. Sci. Nat. in press)

Figure 1. Adult specimen of *Metzneria lappella,* one of the gelechiid moths examined, with long scales along the wing margin.

III. Other research activities

This laboratory also conducts morphological observation of various animal tissues by scanning and transmission electron microscopy and immuno-electron microscopic analyses in collaboration with other laboratories of NIBB. Training in specimen preparation and instrument operation for such observations is also given.

LABORATORY OF BIOLOGICAL DIVERSITY

KAMADA Group

Assistant Professor: *KAMADA,Yoshiaki*

Nutrients are indispensable for life. Among various nutrients amino acids are the major nitrogen source; therefore, perception of the amino acid environment is essential for cells. The cellular amino acid sensing system employs Tor (target of rapamycin) protein kinase. Tor forms two distinct protein complexes, TORC1 (Tor complex1) and TORC2. TORC1 is involved in amino acid sensing, regulating protein synthesis, the cell cycle, and autophagy. On the other hand, TORC2 is responsible for actin organization and cell integrity. So far, it is not clear whether TORC2 also perceives nutrient signals.

The aim of our research group is to reveal the molecular mechanisms of how TORC1 receives amino acid signals and how the TORC1/2 pathways regulate each phenomenon. We have been studying Tor signaling in the budding yeast *Saccharomyces cerevisiae*, and have found three novel branches of the TOR signaling pathways (Figure 1).

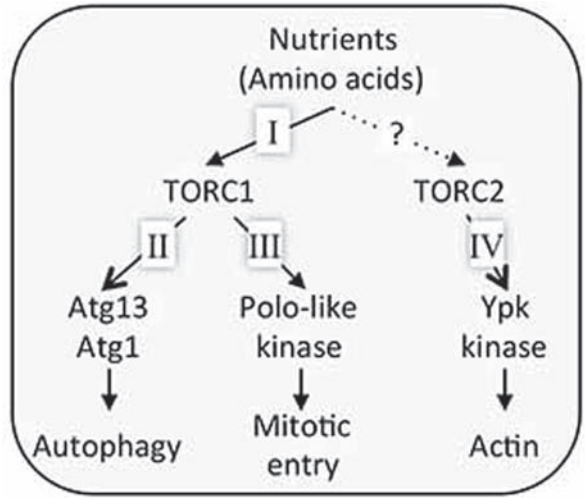


Figure 1. Tor signaling pathway of the budding yeast. Our group has found three branches of the Tor pathway.

I. How do amino acids regulate TORC1?

TORC1 is regulated by amino acids. Amino acids are fundamental nutrients, and 20 species of amino acids building proteins are not interchangeable with each other. Therefore, TORC1 should sense each amino acid individually. Mammalian mTORC1 is believed to be controlled by Rag GTPases and their regulators. However, Rag-factors are dispensable for amino acid sensing by TORC1 in the budding yeast, suggesting an alternative mechanism of TORC1 regulation.

Genetic investigation was done to discover the involvement of (aminoacyl-)tRNA in TORC1 regulation. Biochemical *in vitro* TORC1 assay also revealed that tRNA directly inhibits TORC1 kinase activity. Reducing cellular tRNA molecules desensitizes TORC1 inactivation by nitrogen starvation *in vivo*. Based on these results, a TORC1 regulatory model is proposed that free tRNA released from protein synthesis under amino acid starvation inhibits TORC1 activity. Therefore, TORC1 employs a tRNA-mediated mechanism to monitor intracellular amino acids (Kamada, *in press* 2017).

II. TORC1 phosphorylates Atg13, the molecular switch of autophagy

TORC1 negatively regulates autophagy, a protein degradation system induced by nutrient starvation.

We found the TORC1-mediated regulatory mechanism of autophagy. Under nutrient-rich conditions, TORC1 directly phosphorylates Atg13, a component of the Atg1 kinase complex. Atg1 is a Ser/Thr protein kinase, the activity of which is essential for autophagy and is largely enhanced in response to TORC1 inactivation. Activation of Atg1 requires formation of the Atg1 complex. Phosphorylation of Atg13 by TORC1 plays a pivotal role in Atg1 complex formation; phosphorylated Atg13 loses its affinity to Atg1 resulting in disassembly of the Atg1 complex and repression of autophagy. On the other hand, dephosphorylation of Atg13 triggers formation of the Atg1 complex, activation of Atg1 kinase, and consequently induction of autophagy.

We further determined eight phosphorylation sites of Atg13 and generated an unphosphorylatable Atg13 mutant (Atg13-8SA). Expression of Atg13-8SA induces autophagy bypassing inactivation of TORC1, suggesting that Atg13 acts as a molecular switch for autophagy induction.

III. TORC1 regulates mitotic entry via polo-like kinase

TORC1 regulates protein synthesis, which is important for promotion of the cell cycle at the G1 phase.

We demonstrated that TORC1 is also involved in another stage of the cell cycle, mitotic entry. Cdc5, the yeast polo-kinase mediates this regulation, and the nuclear localization of Cdc5 at G2/M transition is controlled by TORC1. In addition, we discovered a physiological role of TORC1 in mitosis; autophagy negatively controlled by TORC1 plays an important part in maintenance of genome stability under starvation conditions.

IV. Ypk kinase acts directly downstream of TORC2 to control actin organization

TORC2 has an essential function controlling polarity of the actin cytoskeleton.

We found that Ypk2, a member of the AGC kinase family is directly phosphorylated by TORC2. The activated allele of *YPK2* can rescue a lethality caused by TORC2 dysfunction, suggesting that Ypk kinase is the major downstream protein of the TORC2 pathway.

LABORATORY OF BIOLOGICAL DIVERSITY

MANO Group

Assistant Professor:	*MANO, Shoji*
Postdoctoral Fellow:	*KANAI, Masatake*
Visiting Scientist:	*WATANABE, Etsuko*
	KAMIGAKI, Akane
Technical Assistant:	*HIKINO, Kazumi*
	KATO, Kyoko
	NAKAYAMA, Tomomi
Secretary:	*UEDA, Chizuru*

Plant cells can induce, degenerate and differentiate their organelles to adapt to environmental changes. This flexibility of plant organelles is the basis of the strategy for environmental adaptation in plants.

The aims of our research group are to clarify the molecular mechanisms underlying the induction, differentiation, and interaction of organelles, and to understand the integrated functions of individual plants through organelle dynamics.

I. Molecular mechanisms of peroxisome dynamics and functions in plant cells

Peroxisomes are single-membrane bounded organelles, which are ubiquitously present in eukaryotic cells, and they are involved in various biological processes such as lipid metabolism and photorespiration. To understand peroxisome dynamics and functions, we have been analyzing a number of Arabidopsis mutants having aberrant peroxisome morphology (*apem* mutants) and peroxisome unusual poisoning (*peup* mutants). Based on the analyses using these mutants a part of the mechanism of division, protein transport, degradation of peroxisomes, and the interactions of peroxisomes with other organelles were revealed. In addition, we found that peroxisomes are involved in the reproductive process. Therefore, peroxisome dynamics in gametes and gametophytes are currently under investigation.

II. Accumulation mechanism of seed storage oils and proteins

Plant seeds accumulate huge amounts of storage reserves such as oils, carbohydrates and proteins. Humans use these storage reserves as foods and industrial materials. Storage reserves are different among different plant seeds. Wheat, maize and rice seeds mainly accumulate starch, whereas rapeseed, pumpkin and sesame contain large amounts of oils. Soybean contains proteins as a major reserve. We are analyzing the mechanisms controlling oil and protein contents in seeds, and trying to apply our knowledge and techniques for increasing beneficial storage reserves (Figure 1).

III. Construction of The Plant Organelles Database 3 (PODB3)

PODB3 was built to promote a comprehensive understanding of organelle dynamics. PODB3 consists of six individual units: the electron micrograph database, the perceptive organelles database, the organelles movie database, the organellome database, the functional analysis database, and external links. Through these databases, users can obtain information on plant organelle responses to environmental stimuli of various tissues of several plant species, at different developmental stages. We expect that PODB3 will enhance the understanding of plant organelles among researchers.

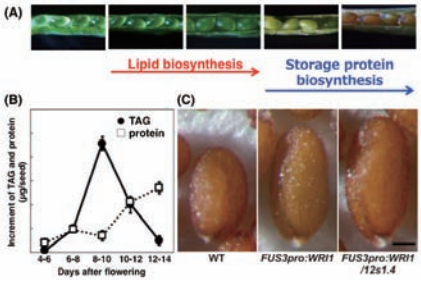


Figure 1. Biosynthesis of lipids and proteins during Arabidopsis seed development and characterization of transgenic seeds overexpressing *WRI1* during the middle and late phases. (A, B) Lipid biosynthesis and accumulation begins before that of seed storage proteins (SSP) during seed development. (C) A master transcription factor regulating seed oil biosynthesis, WRINKLED1 (WRI1), is expressed under the control of the *FUSCA3* (*FUS3*) promoter, which specifically expresses during the middle phase, in the wild type (middle panel) and SSP knockout mutants (right panel). Bar: 0.5 mm.

Publication List:

〔Original papers〕

- Cui, S., Hayashi, Y., Otomo, M., Mano, S., Oikawa, K., Hayashi, M., and Nishimura, M. (2016). Sucrose production mediated by lipid metabolism suppresses physical interaction of peroxisomes and oil bodies during germination of *Arabidopsis thaliana*. J. Biol. Chem. *291*, 19734-19745.
- Hosokawa, Y., Iino, T., Oikawa, K., Mano, S., Yamada, K., and Nishimura, M. (2016). Quantification of the adhesion strength between peroxisomes and chloroplasts by femtosecond laser technology. Bio-Protoc. *6*, e1834.
- Kamigaki, A.*, Nito, K.*, Hikino, K., Goto-Yamada, S., Nishimura, M., Nakagawa, T., and Mano, S. (2016). Gateway vectors for simultaneous detection of multiple protein–protein interactions in plant cells using bimolecular fluorescence complementation. PLoS One *11*, e0160717. (*Co-first authors)
- Kanai, M., Mano, S., Kondo, M., Hayashi, M., and Nishimura, M. (2016). Extension of oil biosynthesis during the mid-phase of seed development enhances oil content in Arabidopsis seeds. Plant Biotechnol. J. *14*, 1241-1250.
- Kimori, Y., Hikino, K., Nishimura, M., and Mano, S. (2016). Quantifying morphological features of actin cytoskeletal filaments in plant cells based on mathematical morphology. J. Theor. Biol. *389*, 123-131.
- Oikawa, K., Mano, S., Yamada, K., Hosokawa, Y., and Nishimura, M. (2016). Measuring the interactions between peroxisomes and chloroplasts by *in situ* laser analysis. Bio-Protoc. *6*, e1790.
- Ueda, H., Yokota, E., Kuwata, K., Kutsuna, N., Mano, S., Shimada, T., Tamura, K., Stefano, G., Fukao, Y., Brandizzi, F., Shimmen, T., Nishimura, M., and Hara-Nishimura, I. (2016). Phosphorylation of the C-terminus of RHD3 has a critical role in homotypic ER membrane fusion in Arabidopsis. Plant Physiol. *170*, 867-880.
- Watanabe, E., Mano, S., Nomoto, M., Tada, Y., Hara-Nishimura, I., Nishimura, M., and Yamada, K. (2016). HSP90 stabilizes auxin-responsive phenotypes by masking a mutation in the auxin receptor TIR1. Plant Cell Physiol. *57*, 2245-2254.

〔Original paper (E-publication ahead of print)〕

- Kanai, M., Mano, S., and Nishimura, M. An efficient method for the isolation of highly purified RNA from seeds for use in quantitative transcriptome analysis. J. Vis. Exp. 2016 Aug 24.

〔Review articles〕

- Kanai, M., Mano, S., Hayashi, M., and Nishimura, M. (2016). Designing novel breeding strategies for producing high-oil crops based on the molecular understanding of triacylglycerol metabolism. In New Challenges in Seed Biology -Basic and translational research driving seed technology-, Araújo, S. and Balestrazzi, A., eds. (Croatia, InTech) pp. 137-153.
- Oikawa, K., Mano, S., Hosokawa, Y., and Nishimura, M. (2016). Analysis of physical interaction between peroxisomes and chloroplast induced by dynamic morphological changes of peroxisomes using femtosecond laser impulsive force. Plant Morphol. *28*, 29-34.

LABORATORY OF BIOLOGICAL DIVERSITY

OHNO Group

Assistant Professor: *OHNO, Kaoru*

The aim of this laboratory is to research reproductive hormones in invertebrates, especially in echinoderms, and to analyze the mechanisms by which they work. The comparisons of such molecules and mechanisms in various species are expected to provide insights into the evolution of reproductive hormone systems.

I. Gonadotropins in the starfish, *Patiria pectinifera*

Gonadotropins play important regulatory roles in reproduction in both vertebrates and invertebrates. The vertebrate gonadotropins, LH and FSH are structurally and functionally conserved across various species, whereas no such molecule has been identified in invertebrates. The insect parsin hormones are assumed to be the physiological counterpart of LH and FSH in mammals. Some gonadotropic hormones, such as the egg development neurosecretory hormone of the mosquito, the egg-laying hormone of the sea hare, and the androgenic gland hormone of the terrestrial isopod, have been found in invertebrate species. More recently, an insulin-like peptide was reported to be responsible for the regulation of egg maturation in the mosquito, *Aedes aegypti*, demonstrating the involvement of insulin signaling in egg maturation among invertebrates.

The gonad-stimulating substance (GSS) of an echinoderm, the starfish, was the very first gonadotropin to be identified in invertebrates. GSS mediates oocyte maturation in starfish by acting on the ovary to produce the maturation-inducing hormone (MIH), 1-methyladenine, which in turn induces the maturation of the oocytes. In this sense, GSS is functionally identical to vertebrate LH, especially piscine and amphibian LHs, acting on the ovarian follicle cells to produce MIH to induce the final maturation or meiotic resumption of the oocyte. Considering the functional similarity that GSS shares with vertebrate LH, it is very important from an evolutionary point of view to know the chemical and molecular structure of GSS. We cloned the gene encoding GSS referred to amino acid sequence of purified GSS from radial nerves of the starfish, *Pateria pectinifera*. Interestingly, phylogenetic analyses revealed that it belonged to the insulin/insulin-like growth factor (IGF)/relaxin superfamily and, more precisely, to the subclass of relaxin peptides (Figure 1).

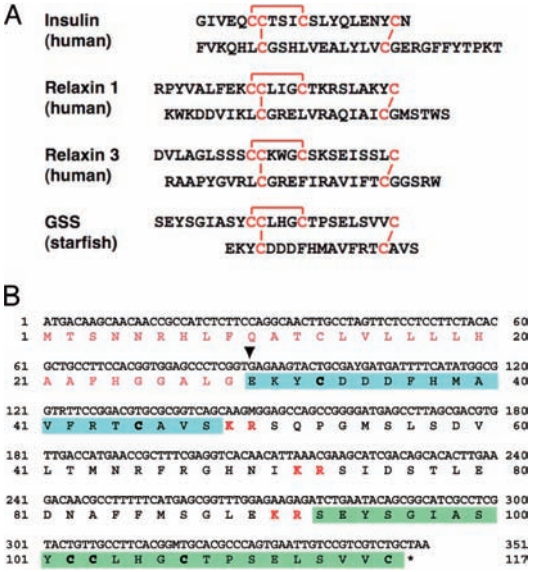


Figure 1. Amino acid sequence of starfish GSS. (A) Comparison of the heterodimeric structure of starfish GSS with those of various representative members of the insulin superfamily. The cysteine bridges are shown in red. (B) Coding DNA sequence and predicted amino acid sequences of GSS. Sequences of A and B chains are shown in green and blue boxes, respectively. Characters shown in red boldface indicate basic dipeptides that are the sites of proteolytic cleavage. Inverted triangle shows the deduced cleavage site of the signal peptide.

II. Search for reproductive hormones in invertebrates

In a collaborative effort with Prof. Yoshikuni's Laboratory of Kyushu Univ. and Dr. Yamano and Dr. Awaji of the National Research Institute of Aquaculture, Fisheries Research Agency (NRIA), we are searching for reproductive hormones in invertebrates; starfishes, brittle stars, sea urchins, sea cucumbers, crinoids, oyster, and shrimp. The collaborators have been able to purify physiological materials which induce egg maturation from nerve extracts and analyze them with a protein sequencer and a tandem mass spectrometer in the analytical center of our institute. One of them, named cubifrin, an NGIWY-amide peptide, in the sea cucumber *Aposticopus japonicus*, the others are in preparation for publications.

We have identified many neuropeptides from our EST analysis of nerve tissues and many from RNA-seq data of the NCBI database. Especially relaxin like peptide precursor genes and insulin/IGF like peptide precursor genes were identified from many species. We are testing production of these neuropeptides by biological methods, e.g. bacterial systems and yeast systems, for providing to collaborators for biological assays.

LABORATORY OF BIOLOGICAL DIVERSITY

KOMINE Group

Assistant Professor: *KOMINE, Yuriko*

We have been interested in the developmental and evolutional aspects of the structure of mammalian brains. In a comprehensive analysis of homeobox genes expressed in the developing mouse neocortex, we isolated a novel gene *Zfhx2*, which encodes a transcription factor containing three homeobox domains and 18 Zn-finger motifs. *Zfhx2* is highly expressed in the developing mouse brain, particularly in differentiating neurons, and continues to be expressed throughout adulthood at a low level. Two other phylogenically related genes, *Zfhx3* and *Zfhx4*, have been identified. The former was reported to be expressed in a manner dependent on neural differentiation, and the latter is a candidate gene causing congenital bilateral isolated ptosis. Although these three genes are expressed in substantially similar patterns in the developing brain, common functional features have not been clarified. Currently we have been focusing on *Zfhx2* to reveal its function and mechanisms of expression control in the developing brain.

I. Expression of *Zfhx2* is negatively regulated by its own antisense RNA

We found that the antisense strand of *Zfhx2* is also expressed in the mouse brain in a manner complementary to the expression of *Zfhx2* mRNA (Figure 1). Although most neurons express *Zfhx2* mRNA immediately after their final mitosis, several types of neuron (e.g., granule cells in the olfactory bulb and pyramidal and granule cells in the hippocampus) express antisense RNA prior to *Zfhx2* mRNA during the early phase of their differentiation. By generating a gene-targeting mouse line in which *Zfhx2* sense RNA is expressed but not antisense RNA, we showed that this antisense RNA has a negative regulatory role in the expression of *Zfhx2* mRNA. These observations suggest that the ZFHX2 protein might have a role in a particular step of neuronal differentiation, and in some types of neuron, this step might be delayed by the expression of antisense RNA.

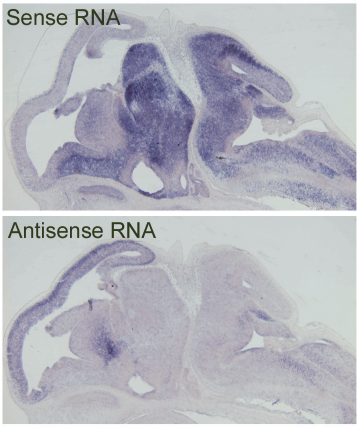


Figure 1. Expression of *Zfhx2* sense RNA (mRNA) and antisense RNA in the embryonic mouse brain. The antisense RNA was expressed where mRNA was not.

II. ZFHX2 might play roles in controlling emotional aspects

To elucidate the function of ZFHX2, we have also generated a *Zfhx2*-deficient mouse line. Although the production of the ZFHX2 protein is completely abolished in the homozygous mutant mice, the mice appear grossly normal and healthy. No anatomical abnormality has been observed in the mutant mouse brains so far examined. We hence subjected the *Zfhx2*-deficient mice to a comprehensive battery of behavioral tests to explore the physiological function of ZFHX2 in the nervous system. The homozygous *Zfhx2* deficient mice showed several behavioral abnormalities, namely, hyperactivity (Figure 2), enhanced depression-like behaviors, and an aberrantly altered anxiety-like phenotype. These behavioral phenotypes suggest that ZFHX2 might play roles in controlling emotional aspects through the function of monoaminergic neurons where ZFHX2 is expressed.

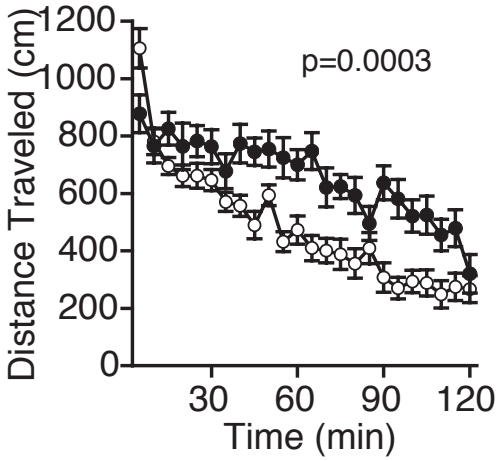


Figure 2. Locomotor activity of the *Zfhx2*-deficient mice. Mice were transferred into a novel environment and the distance traveled of each animal was measured for 2 hours. The *Zfhx2*-deficient mice (●, n=19) were significantly more active than the wild-type mice (○, n=21).

LABORATORY OF BIOLOGICAL DIVERSITY

HOSHINO Group

Assistant Professor: *HOSHINO, Atsushi*
Technical Assistant: *NAKAMURA, Ryoko*
TAKEUCHI, Tomoyo
ITO, Kazuyo

While genomic structures as well as their genetic information appear to stably transmit into daughter cells during cell division, and also into the next generation, they can actually vary genetically and/or epigenetically. Such variability has had a large impact on gene expression and evolution. To understand these genome dynamics in eukaryotes, especially in plants, we are characterizing the flower pigmentation of morning glories including *Ipomoea nil* (Japanese morning glory), *I. purpurea* (the common morning glory), and *I. tricolor*.

I. Flower pigmentation patterns

The wild type morning glories produce flowers with uniformly pigmented corolla, whereas a number of mutants displaying particular pigmentation patterns have been collected. Because flower pigmentation patterns are easily observed, the molecular mechanisms underlying these phenomena provide fine model systems for investigating genome variability.

The recessive mutations, *duskish* of *I. nil* and *pearly-v* of *I. tricolor*, confer variegated flowers, and epigenetic mechanisms are thought to regulate their flower pigmentation. We are currently characterizing detailed molecular mechanisms of these mutations.

II. Genome sequence of *the Japanese morning glory*

To facilitate the studies of our group as well as all morning glory researchers, we conducted *de novo* genome sequencing of *I. nil*. We chose a standard line, Tokyo Kokei Standard, for genome sequencing, and employed shotgun sequencing using a single molecule real time sequencing system. We successfully reported an *I. nil* draft genome sequence with a scaffold N50 of 2.88 Mb, covering 98% of the 750 Mb genome. Scaffolds covering 91% of the genome sequence are anchored to 15 pseudo-chromosomes.

From the draft genome, we could identified around 340 *Tpn1* family transposons, known as the major mutagen of *I. nil*, and found a putative autonomous *Tpn1* transposon, named *TpnA1*. We also identified the *CONTRACTED* gene located on the genetic map published in 1956. Comparative genomic analysis suggested that a whole genome duplication in Convolvulaceae, distinct from the recent whole genome duplication in Solanaceae, has occurred after the divergence of the two sister families.

III. A novel active transposon in the Japanese morning glory

InWDR1 is the multifunction transcriptional regulator of *I. nil*. Characterization of a medicinal herb line showing white flowers and whitish seeds revealed that the line has the *InWDR1* gene carrying an insertion of a *Stowaway*-like transposon, *InSto1*. *InSto1* is the first example of an active transposon inducing spontaneous mutations other than the *Tpn1* family transposons in *I. nil*.

IV. BioResource of morning glories

NIBB is the sub-center for the National BioResource Project (NBRP) for morning glory. In this project, we are collecting, maintaining and distributing standard lines, mutant lines for flower pigmentation, and DNA clones from EST and BAC libraries of *I. nil* and its related species. *I. nil* is one of the most popular floricultural plants in Japan, and has a 100 year history of extensive genetic studies. Our collections include 240 lines and 160,000 DNA clones.

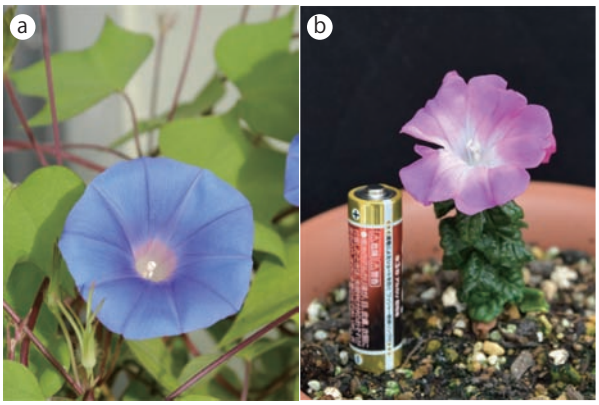


Figure 1. Tokyo Kokei Standard used for genome sequencing (a), and a mutant carrying *contracted* and *star* mutations (b). The *CONTRACTED* and *STAR* genes encode enzymes catalyzing brassinosteroid hormone biosynthesis.

Publication List:

〔Original paper〕

- Azuma, M., Morimoto, R., Hirose, M., Morita, Y., Hoshino, A., Iida, S., Oshima, Y., Mitsuda, N., Ohme-Takagi, M., and Shiratake, K. (2016). A petal-specific InMYB1 promoter from Japanese morning glory: a useful tool for molecular breeding of floricultural crops. Plant Biotechnol. J. *14*, 354-363.
- Hoshino, A., Jayakumar, V., Nitasaka, E., Toyoda, A., Noguchi, H., Itoh, T., Shin-I, T., Minakuchi, Y., Koda, Y., Nagano, A., Yasugi, M., Honjo, M., Kudoh, H., Seki, M., Kamiya, A., Shiraki, T., Carninci, P., Asamizu, E., Nishide, H., Tanaka, S., Park, K.I., Morita, Y., Yokoyama, K., Uchiyama, I., Tanaka, Y., Tabata, S., Shinozaki, K., Hayashizaki, Y., Kohara, Y., Suzuki, Y., Sugano, S., Fujiyama, A., Iida, S., and Sakakibara, Y. (2016). Genome sequence and analysis of the Japanese morning glory *Ipomoea nil*. Nat. Commun. *7*, 13295.
- Hoshino, A., Yoneda, Y., and Kuboyama, T. (2016). A *Stowaway* transposon disrupts the *InWDR1* gene controlling flower and seed coloration in a medicinal cultivar of the Japanese morning glory. Genes Genet. Syst. *91*, 37-40.

LABORATORY OF BIOLOGICAL DIVERSITY

TSUGANE Group

Assistant Professor: *TSUGANE, Kazuo*

Although transposons occupying a large portion of the genome in various plants were once thought to be junk DNA, they play an important role in genome reorganization and evolution. Active DNA transposons are important tools for gene functional analysis. The endogenous non-autonomous transposon, *nDart1-0*, in rice (*Oryza sativa* L.) is expected to generate various transposon-insertion mutants because *nDart1-0* elements tend to insert into genic regions under natural growth conditions. The transpositions of *nDart1-0* were promoted by an active autonomous element, *aDart1-27*, on chromosome 6. By using the endogenous *nDart1*/*aDart1-27* system in rice, a large-scale *nDart*-inserted mutant population was easily generated under normal field conditions, and the resulting tagged lines were free of somaclonal variation. The *nDart1*/*aDart1-27* system was introduced into a rice variety, Koshihikari, named MK-1. 3000 MK-1 plants were grown in field conditions (IPSR, Okayama Univ.) (Figure 1). The genome of all plants were isolated for identifying insertion sites of *nDart1*.

Figure 1. Mk-1 plants were grown at normal field condition. Various mutants were observed.

I. Semidominant mutation in rice

The semidominant mutations produce the intermediate phenotype in individuals heterozygous for the gene concerned. The semidominant mutations were occasionally isolated from the MK-1, it was unclear what causes dominant mutations. Efficient selection and analysis of dominant mutants to analyze the gene functions in rice is very useful. Newly isolated, *Bushy dwarf tiller2* (*Bdt2*), which has the valuable agronomic traits of multiple tillering and dwarfism, was obtained from the MK-1 (Figure 2). Genetic analysis revealed the *Bdt2* mutation was controlled by two genetic elements. One *Bdt2* element, *Bdt2a* showed weak dwarf phenotype, another element, *Bdt2b* strengthened the effect of *Bdt2a*. A wild plant produces only 10 spikes or less, while *Bdt2a* produced 3 times as many, *Bdt2* produced 10 times the ears.

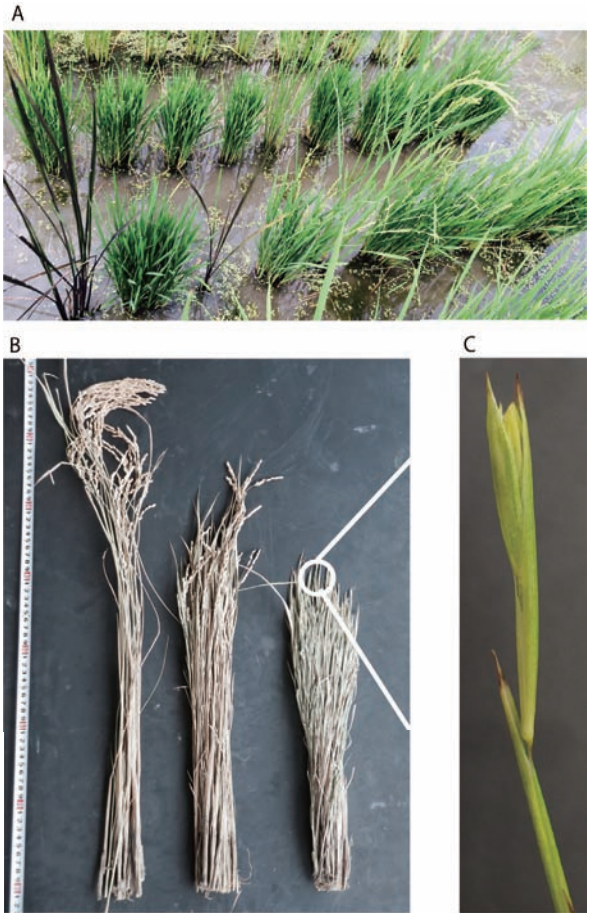


Figure 2. Phenotype of *Bushy dwarf tillers2* (*Bdt2*). (A) Two-month-old plants in the field (B) Segregants of *Bdt2* mutants. *Wild type* (left), *Bdt2a* (middle), *Bdt2*(right). (C) Abnormal bract of *Bdt2*.

Publication List:

〔Original paper〕

● Gichuhi, E., Himi, E., Takahashi, H., Zhu, S., Doi, K., Tsugane, K., and Maekawa, M. (2016). Identification of QTLs for yield-related traits in RILs derived from the cross between pLIA-1 carrying *Oryza longistaminata* chromosome segments and Norin 18 in rice. Breed. Sci. *66*, 720-733.

LABORATORY OF BIOLOGICAL DIVERSITY

JOHZUKA Group

Assistant Professor: *JOHZUKA, Katsuki*
Technical Assistant: *ISHINE Naomi*

Chromosome condensation is a basic cellular process that ensures the faithful segregation of chromosomes in both mitosis and meiosis. This process is required not only for shrinking the length of chromosome arms, but also for resolving entanglements between sister-chromatids that are created during DNA replication. Any abnormality in this process leads to segregation errors or aneuploidy, resulting in cell lethality. Chromosome condensation is mainly achieved by condensin, a hetero-pentameric protein complex, widely conserved from yeast to humans. Despite its conservation and importance for chromosome dynamics, how condensin works is not well understood. Recent studies reveal that condensin functions are not restricted to chromosome condensation and segregation during cell divisions. It is required for diverse DNA metabolism such as genome stability, transcriptional regulation, and cell differentiation.

Our research interest is to understand the mechanism and regulation of chromosome condensation. We have been studying the role of condensin in the budding yeast *Saccharomyces cerevisiae*. Microscopic observation indicated the nucleolar localization of condensin. Consistent with this, the ribosomal RNA gene (rDNA) repeat is the most condensed region in the genome during mitosis. We have found that condensin specifically binds to the RFB site located within the rDNA repeat. To date, the best characterized condensin binding region is the rDNA repeat on the right arm of chromosome XII in budding yeast. We further discovered the multiple protein network required to recruit condensin to the RFB site.

I. Dynamic relocalization of condensin during meiosis

Our genetic screening indicated that two proteins, Csm1 and Lrs4, were required for condensin recruitment to the RFB site. Physical interactions between Csm1/Lrs4 and subunits of condensin are important for recruitment of condensin to the RFB site. These proteins are known as components of monopolin complex that are required for faithful segregation of homologous chromosomes during meiotic division I. During meiosis I, monopolin complex re-localizes from rDNA repeat to the centromere and acts for ensuring sister-chromatid co-orientation. Re-localization of Csm1/Lrs4 proteins suggested the re-localization of condensin from rDNA repeat to centromere. As expected, chromatin-IP experiments indicated that condensin re-localizes to the centromere during meiosis I. Condensin might clamp sister-chromatids together during meiosis I.

II. Condensin-dependent chromatin folding

The RFB site, which consists of a ~150bp DNA sequence, is functioning as a cis-element for recruitment of condensin to chromatin in the yeast genome. If the RFB site is inserted into an ectopic chromosomal locus, condensin can associate with the ectopic RFB site. To explore the role of condensin in chromosome organization, we have constructed a strain in which two RFB sites are inserted on an ectopic chromosome arm with an interval of 15kb distance in the cell with complete deletion of chromosomal rDNA repeat. Using this strain, condensin-dependent chromatin interaction between two RFBs was examined by chromosome conformation capture (3C) assay. We found the condensin-dependent chromatin interaction between the two RFB sites on the chromosome arm. This result indicates that condensin plays a role in chromatin interaction between condensin binding sites, and this interaction leads to creation of a chromatin loop between those sites (Figure 1). It is thought that condensin-dependent chromatin folding is one of the basic molecular processes of chromosome condensation. During the cell cycle stages, the RFB - RFB interaction signal increases in metaphase and reaches its maximum level in anaphase. In addition to the RFB – RFB interaction, the chromatin interactions between internal regions of two RFBs increases in anaphase. Thus, the configuration of chromatin fiber changes from a simple loop into a complicated twisted shape as the cell cycle progresses from metaphase to anaphase.

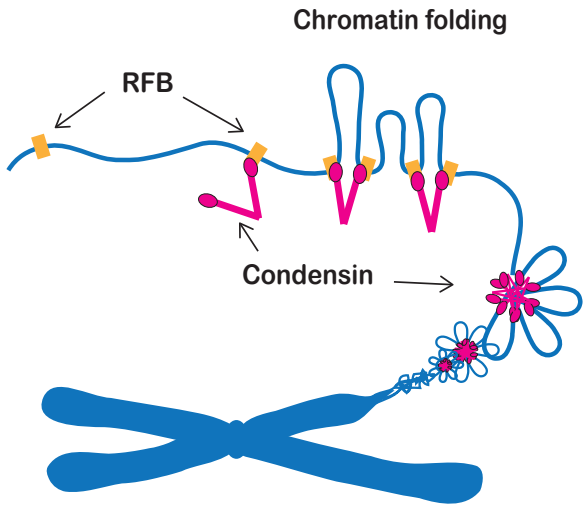


Figure 1. A Schematic model of chromosome condensation. Condensin makes chromatin interactions between adjacent binding sites (RFB, for example). This leads to a folding of chromatin fibers between the sites, as a basic process of chromosome condensation.

LABORATORY OF BIOLOGICAL DIVERSITY

KATO Group

Specially Appointed Assistant Professor:
KATO, Kagayaki

Organogenesis is accomplished by a series of deformations of the planar cell sheet into a three-dimensional shape during embryogenesis. This drastic structural change is an integrated result of individual cell behaviors in response to spatio-temporally controlled mechanisms.

To better understand the programs underlying organ formation, it is required to analyze individual cells' morphology and dynamics quantitatively. However, due to the massive images generated by 4D microscopy and their ambiguity, this made it difficult to perform these analyses.

To unveil organogenesis from the point of view of distinct cell behaviors, we are developing application software that is capable of describing cell dynamics out of 4D time-lapse imaging data sets by employing image processing techniques.

I. 4D cell segmentation/tracking system

Epithelial morphogenesis in the developing embryo is considered to be an essential model for collective cell migrations. Drastic cell rearrangements lead drastic structural changes to build elaborate organs such as the tubular network of *Drosophila* trachea. We are developing a software pipeline, which automatically recognizes individual cell shapes out of 3D space and tracks them through time. This system extracts cell boundaries and reconstitutes cell shapes as a skeletonized chain of voxels spanning 3D space. This abstract form of cell visualization makes it possible to describe morphometric quantities and kinetics of cells in single-cell resolution (Figure 1). These morphometric quantities allow us to perform comparative studies on shapes and behaviors precisely among several experimental conditions, to gain a better understanding of the genetic programs underlying organogenesis. We are now applying this system to several experimental models to determine the practicality of the system.

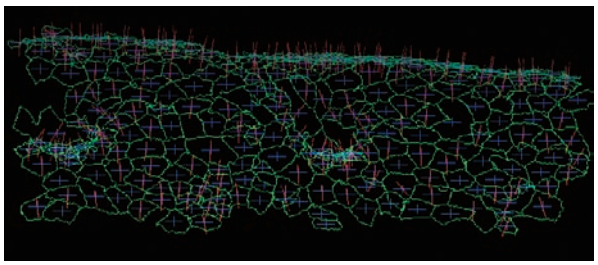


Figure 1. Visualized apical cell surface of *Drosophila* embryonic epidermal cells. A time-lapse confocal microscopic data set of a fly embryo expressing E-cadherin-GFP was subjected to our automatic cell shape recognition system. Cell boundaries (green), center of gravity (blue) and normal vector (magenta) are indicated for each cell.

II. Particle tracking for tissue deformation analysis

Besides cell boundary extraction, we also developed a derived algorithm for particle image velocimetry (PIV). This system is designed to measure tissue deformation even though the imaging constraints do not allow identification of individual cells out of images. This implementation detects structural characteristics, such as uneven fluorescence distributed over the specimen and tracks these patterns along a time-series. Despite that the tissue was labeled with non-targeted cytoplasmic GFP, this tracking software successfully outlined developmental dynamics of *Xenopus* neuroectoderm (Figure 2).

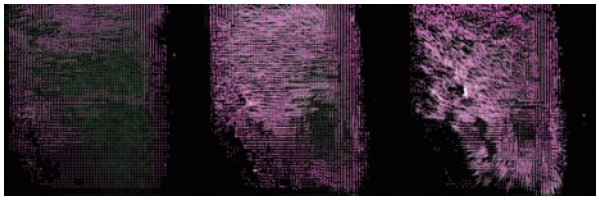


Figure 2. Collective cell migration of *Xenopus* neuroectodermal cells visualized as optical flow along a time-series. A modified PIV method successfully tracks uneven subcellular distribution of GFP signals over time. Dr. M. Suzuki (Prof. Ueno's laboratory at NIBB) performed the microscopy.

III. A GUI application for manual image quantification

Biologically significant image features are not always significant to computational algorithms due to their structural instability. This kind of difficulty requires human eye inspection for feature extractions. A GUI (Graphical User Interface) application we developed can easily visualize 4D imaging data and has made manual feature annotations easy (Figure 3). This application is freely available at our website (https://is.cnsi.nins.jp/).

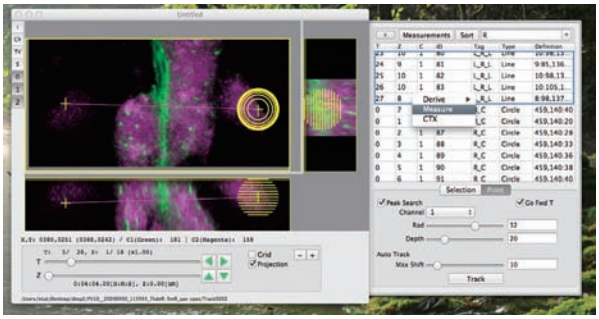


Figure 3. A lightweight/native 4D stack viewer equipped with functions for manual feature extraction.

Publication List:

〔Original Paper〕

● Kato, K., Dong, B., Wada, H., Tanaka-Matakatsu, M., Yagi, Y., and Hayashi, S. (2016). Microtubule-dependent balanced cell contraction and luminal-matrix modification accelerate epithelial tube fusion. Nat. Commun. *7*, 11141.

LABORATORY OF BIOLOGICAL DIVERSITY

KIMORI Group

Specially Appointed Assistant Professor:
KIMORI, Yoshitaka

Image processing methods significantly contribute to visualization of biomedical targets acquired from a variety of imaging techniques, including: wide-field optical and electron microscopy, X-ray computed tomography, magnetic resonance imaging and mammography. Quantitative interpretation of the deluge of complicated biomedical images, however, poses many research challenges. We have developed new computational methods based on mathematical morphology for quantitative image analysis. One of the most important purposes of image processing is to derive meaningful information, which is expressed as image structural properties. Mathematical morphology is a nonlinear image processing method based on set theory and is useful for the extraction of the structural properties from an image. It can be used as a fundamental tool to analyze biomedical images.

Novel image segmentation method based on mathematical morphology

Image processing is a crucial step in the quantification of biomedical imaging data. As such, it is fundamental to a wide range of biomedical imaging fields. Image processing derives structural features, which are then numerically quantified by image analysis. Image segmentation plays an important role in image processing. Segmentation is the partitioning of an image into sub-regions and the extraction of a target that is to be analyzed.

In this study, an image segmentation approach based on a new type of mathematical morphology is introduced. Mathematical morphology is a methodology for extracting shape and size information from an image. It involves configuration of a set of nonlinear operators that act on images by using structuring elements (SE). The SE, which indicates the shape characteristics in an image, is generally a small and simple binary image. The two basic morphological operators are dilation and erosion, from which many operations can be derived.

This segmentation method is based on a double morphological subtraction method. Generally, morphological subtraction methods may include top-hat transform, rolling-ball transform, and h-maxima transform. These methods extract target objects that are brighter than the surrounding areas. However, these methods may also extract unwanted structures which are located in the neighborhood of the target object. Thus, an identification process between the target regions and other object regions is required. Commonly, this task is difficult due to intensity and size similarities among these extracted regions.

In this new type of morphological segmentation method, h-maxima transform is applied twice to the original image. The unwanted structures that surround the target are suppressed in the process of target segmentation. Furthermore, this new method has no restrictions on the size and shape of the target object that is to be segmented.

This method was applied to segmentation of an abnormal region in the chest x-ray image shown in Fig. 1. The arrow in Fig. 1(a) shows the position of the abnormality (lung nodule). Fig. 1(b) shows the image after segmentation using the proposed method. Candidates of lung nodule regions were extracted. Fig. 1(c) is the resultant image of nodule detection. The nodule region was clearly distinguished from the surrounding tissues.

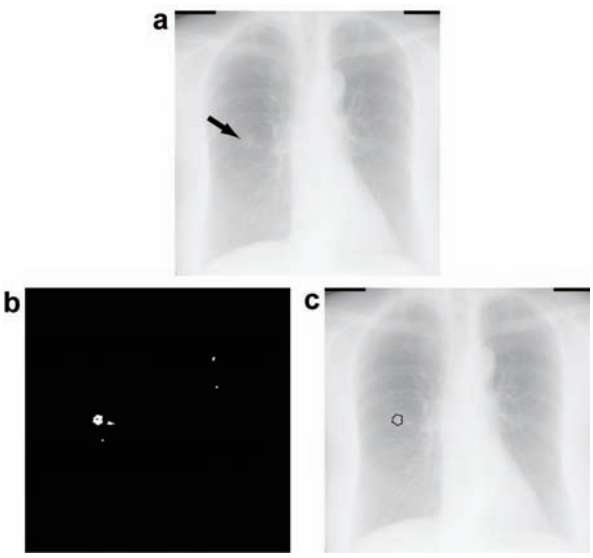


Figure 1. Segmentation results for chest x-ray image: (a) test image. (b) Segmentation using the proposed method. The image was obtained from a standard digital image database of chest lung nodules and non-nodules (Shiraishi et al., *ARJ Am. J. Roentgenol*. 174, 2000). (a) Original chest x-ray image. (b) Segmentation result of candidates of lung nodule obtained by the proposed method. (c) Detection of lung nodule.

Publication List:

〔Original paper〕

● Murata, K., Hagiwara, S., Kimori, Y., and Kaneko, Y. (2016). Ultrastructure of compacted DNA in cyanobacteria by high-voltage cryo-electron tomography. Sci. Rep. *6*, 34934.

〔Original paper (E-publication ahead of print)〕

● Osanai, Y., Shimizu, T., Mori, T., Yoshimura, Y., Hatanaka, N., Nambu, A., Kimori, Y., Koyama, S., Kobayashi, K., and Ikenaka, K. Rabies virus-mediated oligodendrocyte labeling reveals a single oligodendrocyte myelinates axons from distinct brain regions. Glia 2016 Oct 19.

DIVISION OF MOLECULAR ENVIRONMENTAL ENDOCRINOLOGY †

Professor
IGUCHI, Taisen

Assistant Professor:	*MIYAGAWA, Shinichi*
	OGINO, Yukiko
Technical Staff:	*MIZUTANI, Takeshi*
NIBB Research Fellow:	*TOYOTA, Kenji*
Postdoctoral Fellow:	*MIYAKAWA(OKAMOTO), Misato*
SOKENDAI Graduate Student:	*SUMIYA, Eri*
	YATSU, Ryohei
Visiting Graduate Student:	*TOHYAMA, Saki*
Technical Assistant:	*HAYASHI, Tomoko*
	INABA, Kayo
Secretary:	*IMAIZUMI, Taeko*

Synthetic chemicals found in the environment have the capacity to disrupt the development and function of the endocrine system in both wildlife and humans. This has drawn public concern since many of these chemicals may bind to estrogen receptors (ERs) and evoke estrogenic effects. Early evidence that exposure to estrogenic chemicals during development could pose a threat to human health came from studies of a synthetic hormone, diethylstilbestrol (DES), which was used to prevent premature birth and spontaneous abortion. Laboratory experiments showed that exposure to sex hormones during critical windows of perinatal life caused the immune and nervous systems, bone, muscle, and the liver of animals to be affected. Although many of these chemicals can bind to ERs in wildlife and humans, the molecular basis for the action of environmental estrogens remains poorly understood. Thus, understanding the molecular mechanisms through which environmental estrogens and sex hormones act during critical developmental windows is essential.

I. Developmental origin of adult disease: Perinatal estrogen exposure induces persistent changes in reproductive tracts

The emerging paradigm of the "embryonic/fetal origins of adult disease" provides a powerful new framework for considering the effects of endocrine disrupters on human and animal health. In 1971, prenatal DES exposure was found to result in various abnormalities of the reproductive tract in women. This syndrome was named the DES syndrome. Similar abnormalities have been demonstrated in experimental animals exposed perinatally to estrogens. Developmental estrogen exposure in mice, for example, induces persistent proliferation of vaginal epithelial cells. We found that the persistent changes in the vagina in mice exposed neonatally to estrogens result from the persistent activation of erbBs and ERα, and sustained expression of EGF-like growth factors. Currently, we are analyzing the methylation status in the mouse vagina using a microarray (MeDIP-chip). We found several differentially methylated or demethylated DNA profiles in neonatally DES-exposed mouse vaginae and controls. We thus consider that neonatal DES exposure affects DNA methylation profiles, resulting in persistent abnormalities in mouse reproductive organs. We also found that ERα is indispensable for normal vaginal epithelial cell differentiation in mice, and retinoic acid signaling determines the fate of uterine stroma in the mouse Müllerian duct.

II. Estrogen receptors of birds, reptiles, amphibians, and fishes

Steroid and xenobiotic receptors (SXR) have been cloned from various animal species (fish, amphibians, reptiles, birds, and mammals) by our group and we have demonstrated species-specific differences in their responses to various environmental and endogenous chemicals (receptor gene zoo). Thus, simple predictions of chemical effects based on data from a few established model species are not sufficient to develop real world risk assessments. ER and ER-like genes have been cloned from various animal species including rockshell, *Amphioxus*, lamprey, catshark, whale shark, lungfish, sturgeon, gar, *polypterus*, arowana, roach, stickleback, mosquitofish, mangrove *Rivulus*, Japanese giant salamander, Tokyo salamander, newt, axolotl, toad, *Silurana tropicalis*, American alligator, Nile crocodile, freshwater turtle, Japanese rat snake, Okinawa habu, and vultures. Functional studies showed that the *Amphioxus* ER sequence does not bind estrogen but *Amphioxus* steroid receptor and lamprey ER exhibited ligand-dependent transactivation, proving that invertebrate and primitive vertebrates, such as the Agnatha, have a functional ER. We found that medaka ER subtypes have their specific functions, and medaka, zebrafish and stickleback ERs are more sensitive to estrogen/estrogen-like chemical exposures than other fishes by reporter gene assay. Squamae ER is the most sensitive in animal species. Thus, these approaches are efficient to evaluate the relationship between species and their sensitivities to chemicals.

III. Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates

Vertebrates show diverse sexual characteristics which are regulated by androgens. To elucidate the evolutionary history and functional diversification of androgen receptor (AR) genes in vertebrates, we cloned the AR cDNAs from a shark, basal ray-finned fishes (Actinopterygii), namely bichir and sturgeon (Acipenseriformes), and teleosts including a basal teleost, arowana (Osteoglossiformes). Molecular phylogenetic analysis revealed that a gene duplication event gave rise to two different teleost ARs (α and β) and likely occurred in the actinopterygian lineage leading to teleosts after the divergence of Acipenseriformes but before the split of Osteoglossiformes. Functional analysis revealed that the shark AR activates the target gene via the androgen response element by classical androgens. The teleost ARα showed unique intracellular localization with a significantly higher transactivation capacity than that of teleost ARβ. Therefore, the most ancient type of AR emerged before the Chondrichthyes-Osteichthyes split and the *AR* gene was duplicated during a teleost-specific gene duplication event.

†: This laboratory was closed on 31 March, 2016.

VI. Environmental sex differentiation in Daphnids and American alligators

Daphnia magna has been used extensively to evaluate the organism- and population-based responses to toxicity or reproductive toxicity tests. These tests, however, provide no information about the mode of action of the tested compounds. Therefore, we applied an ecotoxicogenomic assessment of *D. magna*. *D. magna* and *D. pulex* reproduce asexually (parthenogenesis) when they are in an optimal environment for food, photoperiod and population density. Once environmental conditions become sub-optimal, they alter their reproductive strategy from asexual to sexual reproduction. Chemicals are able to affect the sex determination of daphnids and we found that juvenile hormone (JH) agonists (insect growth regulators), for example, induce the production of male offspring. The molecular basis of environmental sex determination is largely unknown in daphnids. To understand the molecular mechanisms of this phenomenon, we isolated sex determination-related genes. Also, we have developed a method to inject genes into *D. magna* and *D. pulex* embryos which will allow us to study gain- and loss-of function analyses in more detail in these species. Using these techniques, we demonstrated that DSX1 (double sex 1), one of the DM-domain genes, is essential for male differentiation in *D. magna*. We have developed an RNAi method and a TALEN method using *D. pulex*. To further explore the signaling cascade of sexual differentiation in *D. magna,* a gene expression profile of JH-responsive genes is essential. We identified JH-responsive genes in the ovary of *D. magna* and *D. pulex* exposed to JH agonist and methyl farnesoate (JH identified in decapods) at the critical timing of JH-induced sex determination in *D. magna* and *D. pulex*. We have identified a JH receptor (heterodimer of methoprene-tolerant and steroid receptor co-activator) in daphnids and the function of ecdysone in molting and ovulation in *D. magna*. We established a reliable induction system of female and male offspring in *D. pulex* WTN6 strain in response to photoperiod differences, and reported developmental staging of female and male *D. pulex* and *D. magna* during embryogenesis. Metabolomics revealed involvement of pantothenate for male production responding to the short-day stimulus, and also protein kinase C also has an essential role in induction of males in *D. pulex*

Sex determination mechanisms can be broadly categorized by either a genotypic or environmentally driven mechanism. Temperature-dependent sex determination (TSD), an environmental sex determination mechanism most commonly observed among vertebrates, has been observed especially among reptiles from 1966. However, the temperature-dependent triggering mechanism of TSD and the subsequent differentiation cascade has long remained unknown. We have isolated and cloned the thermosensitive cation channel, TRP vanilloid subtype 4 (TRPV4) as a male-cascade trigger for the American alligator, *Alligator mississippiensis*, in response to high environmental temperature, and demonstrated its thermal activation at temperatures proximate to TSD-related temperatures in the alligator. Furthermore, using pharmacological exposure to manipulate TRPV4 channel activity, we have demonstrated that TRPV4 channel activity has a direct relationship with male differentiation gene expression, suggesting that AmTRPV4 is involved in the male differentiation cascade, and proposed a novel mechanism for the sex determination pathway.

Publication List:

〔Original papers〕

- Chakraborty, T., Zhou, L.Y., Chaudhari, A., Iguchi, T., and Nagahama, Y. (2016). Dmy initiates masculinity by altering Gsdf/Sox9a2/Rspo1 expression in medaka (*Oryzias latipes*). Sci. Rep. *6*, 19480.
- Haselman, J.T., Kosian, P.A., Korte, J.J., Olmstead, A.W., Iguchi, T., Johnson, R.D., and Degitz, S.J. (2016). Developemnt of the larval amphibian growth and development assay: Effects of chronic 4-*tert*-octylphenol or 17β-trenbolone exposure in *Xenopus laevis* from embryo to juvenile. J. Appl. Toxicol. *36*, 1639-1650.
- Haselman, J.T., Sakurai, M., Watanabe, N., Goto, Y., Onishi, Y., Ito, Y., Onoda, Y., Kosian, P.A., Korte, J.J., Johnson, R.D., Iguchi, T., and Degitz, S.J. (2016). Development of the larval amphibian growth and development assay: Effects of benzophenone-2 exposure in *Xenopus laevis* from embryo to juvenile. J. Appl. Toxicol. *36*, 1651-1661.
- Katsu, Y., Cziko, P.A., Chandsawangbhuwana, C., Thornton, J.W., Sato, R., Oka, K., Takei, Y., Baker, M.E., and Iguchi, T. (2016). A second estrogen receptor from Japanese lamprey (*Lethenteron japonicum*) does not have activities for estrogen binding and transcription. Gen. Comp. Endocrinol. *236*, 105-114.
- Nakajima, T., Iguchi, T., and Sato, T. (2016). Retinoic acid signalling determines the fate of uterine stroma in the mouse Müllerian duct. Proc. Natl. Acad. Sci. USA *113*, 14354-14359.
- Ogino, Y., Kuraku, S., Ishibashi, H., Sumiya, E., Miyagawa, S., Matsubara, H., Yamada, G., Baker, M.E. and Iguchi, T. (2016). Neofunctionalization of androgen receptor by gain-of-function mutations in teleost fish lineage. Mol. Biol. Evol. *33*, 228-244.
- Oka, K., Kohno, S., Ohta, Y., Guillette, L.J. Jr., Iguchi, T., and Katsu, Y. (2016). Molecular cloning and characterization of the aryl hydrocarbon receptors and aryl hydrocarbon receptor nuclear translocators in the American alligator. Gen. Comp. Endocrinol. *238*, 13-22.
- Song, Y., Rundberget, J.T., Xie, L., Gomes, T.C., Høgåsen, T., Iguchi, T., and Tollefsen, K.E. (2016). Whole-organism transcriptomic analysis provides mechanistic insight into the acute toxicity of emamectin benzoate in *Daphnia magna*. Environ. Sci. Technol. *50*, 11994–12003.
- Sumiya, E., Ogino, Y., Toyota, K., Miyakawa, H., Miyagawa, S., and Iguchi, T. (2016). *Neverland* regulates embryonic moltings through the regulation of ecdysteroid synthesis in the water flea *Daphnia magna*, and may thus act as a target for chemical disruption of molting. J. Appl. Toxicol. *36*, 1476-1485.
- Terauchi, K.J., Shigeta, Y., Iguchi, T., and Sato, T. (2016). Role of Notch signaling in granulosa cell proliferation and polyovular follicle induction during folliculogenesis in mouse ovary. Cell Tissue Res. *365*, 197-206.
- Tohyama, S., Miyagawa, S., Lange, A., Ogino, Y., Mizutani, T., Ihara, M., Tanaka, H., Tatarazako, N., Kobayashi, T., Tyler, C.R., and Iguchi, T. (2016). Evolution of estrogen receptors in ray-finned fish and their comparative responses to estrogenic substances. J. Steroid Biochem. Mol. Biol. *158*, 189-197.
- Toyota, K., Gavin, A., Miyagawa, S., Viant, M.R., and Iguchi, T. (2016). Metabolomics analysis reveals an involvement of pantothenate in male offspring production in response to the short-day stimulus in the water flea, *Daphnia pulex*. Sci. Rep. *6*, 25125.
- Toyota, K., Hiruta, C., Ogino, Y., Miyagawa, S., Okamura, Y., Onishi, Y., Tatarazako, N., and Iguchi, T. (2016). Comparative developmental staging of the female and male water fleas *Daphnia pulex* and *Daphnia magna* during embryogenesis. Zool. Sci. *33*, 31-37.
- Toyota, K., McNabb, N.A., Spyropoulos, D.D., Iguchi, T., and Kohno, S. (2016). Toxic effects of chemical dispersant Corexit 9500 on water flea *Daphnia magna*. J. Appl. Toxicol. *37*, 201-206.
- Yatsu, R., Katsu, Y., Kohno, S., Mizutani, T., Ogino, Y., Guillette, L.J. Jr., Miyagawa, S., and Iguchi, T. (2016). Characterization of evolutionary trend in squamate estrogen receptor sensitivity. Gen.

Comp. Endocrinol. *238*, 88-95.

- Yatsu, R., Miyagawa, S., Kohno, S., Parrott, B.B., Yamaguchi, K., Ogino, Y., Miyakawa, H., Lowers, R.H., Shigenobu, S., Guillette, L.J. Jr., and Iguchi, T. (2016). RNA-seq analysis of the gonadal transcriptome during *Alligator mississippiensis* temperature-dependent sex determination and differentiation. BMC Genomics *17*, 77.

〔Review article〕

- Zoller, T., Bergman, Å., Becher, G., Bjerregaard, P., Bornman, R., Brandt, I., Iguchi, T., Jobling, S., Kidd, K., Kortenkamp, A., Slallaebaek, N., Toppari, J., and Vandenberg, L. (2016). The path forward on endocrine disruptors requires focus on the basics. Toxicol. Sci. *149*, 272.

DIVISION OF ENVIRONMENTAL PHOTOBIOLOGY

Professor
MINAGAWA, Jun

Associate Professor
TAKAHASHI, Shunichi

Assistant Professor:	*TOKUTSU, Ryutaro*
Technical Staff:	*NODA, Chiyo*
NIBB Research Fellow:	*AIHARA, Yusuke*
Postdoctoral Fellow:	*KAMADA, Konomi*
	YAMASAKI, Tomohito
	KAWAI, Hisako
	YAMASAKI, Hiroaki
	KIM, Eunchul
SOKENDAI Graduate Student:	
	KAMRANI, Yousef Yari
	KATO, Hiroki
	KOSUGE, Kotaro
	WATANABE, Akimasa
	OKAJIMA, Keisuke
Visiting Scientist:	*TAKIZAWA, Kenji*
	TAKAHASHI, Sara
Technical Assistant:	*YONEZAWA, Harumi*
	KADOWAKI, Tamaka
	KIDA, Emi
Secretary:	*KOJIMA, Yoko*

Plants and algae have a large capacity to acclimate themselves to changing environments. We are interested in these acclimation processes, in particular, how efficiently yet safely they harness sunlight for photosynthesis under fluctuating light conditions. Using a model green alga, we are studying the molecular mechanisms underlying photoacclimation of the photosynthetic machinery. We are also applying the knowledge obtained in the studies of a model green alga to various phytoplankton, including *Symbiodinium* in corals and sea anemones in tropical oceans, to explore how environmentally important photosynthetic organisms thrive in their ecological niche.

I. Non-photochemical quenching

Absorption of light in excess of the capacity for photosynthetic electron transport is damaging to photosynthetic organisms. Several mechanisms exist to avoid photodamage, which are collectively referred to as non-photochemical quenching (NPQ). This term comprises at least two major processes: state transitions (qT), the change in the relative antenna sizes of PSII and PSI, and energy-dependent quenching of excess energy (qE), the increased thermal dissipation triggered by lumen acidification. Recently, we isolated the PSII-LHCII supercomplex from both WT *C. reinhardtii* and the *npq4* mutant, which is qE-deficient and lacks the ancient light-harvesting protein LHCSR. LHCSR3 was present in the PSII-LHCII supercomplex from the high light-grown WT but not in the supercomplex from the low light-grown WT or the *npq4* mutant. The purified PSII-LHCII supercomplex containing LHCSR3 showed a normal fluorescence lifetime at a neutral pH (7.5) by single-photon counting analysis but exhibited a significantly shorter lifetime (energy-quenching) at pH 5.5, which mimics the acidified lumen of the thylakoid membranes in high light-exposed chloroplasts. The switching from light-harvesting mode to energy-dissipating mode observed in the LHCSR3-containing PSII-LHCII supercomplex was inhibited by DCCD, a protein-modifying agent specific to protonatable amino acid residues. We conclude that the PSII-LHCII-LHCSR3 supercomplex formed in high light-grown *C. reinhardtii* cells is capable of energy dissipation upon protonation of LHCSR3. However, the environmental cue that triggers the expression of LHCSR3 protein has been elusive.

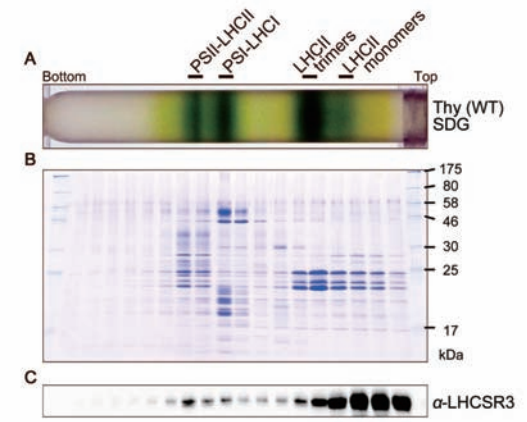


Figure 1. Purification of the PSII-LHCII-LHCSR3 supercomplex from WT *C. reinhardtii*. (A) Thylakoids from WT cells grown under high light conditions were subjected to sucrose density gradient centrifugation (SDG). (B) Polypeptides in the SDG fractions shown in (A) were analyzed by SDS/PAGE. (C) Polypeptides in the SDG fractions were subjected to immunoblotting with an antibody against LHCSR3.

In plants and algae, light serves both as the energy source for photosynthesis and as a biological signal that triggers cellular responses via specific sensory photoreceptors. Red light is perceived by bilin-containing phytochromes and blue light by the flavin-containing cryptochromes and/or phototropins (PHOTs), the latter containing two photosensory light, oxygen, or voltage (LOV) domains. Photoperception spans several orders of light intensity, ranging from far below the threshold for photosynthesis to values beyond the capacity of photosynthetic CO_2 assimilation. We revealed that PHOT controls qE by inducing the expression of LHCSR3 in high light intensities. This control requires blue-light perception by LOV domains on PHOT, LHCSR3 induction through PHOT kinase, and light dissipation in photosystem II via LHCSR3. Mutants deficient in the PHOT gene display severely reduced fitness under excessive light conditions, indicating that the sensing, utilization, and dissipation of light is a concerted process that plays a vital role in microalgal acclimation to environments of variable light intensities. Here we demonstrated the existence of a molecular link between photoreception, photosynthesis, and photoprotection in the green alga *Chlamydomonas reinhardtii*.

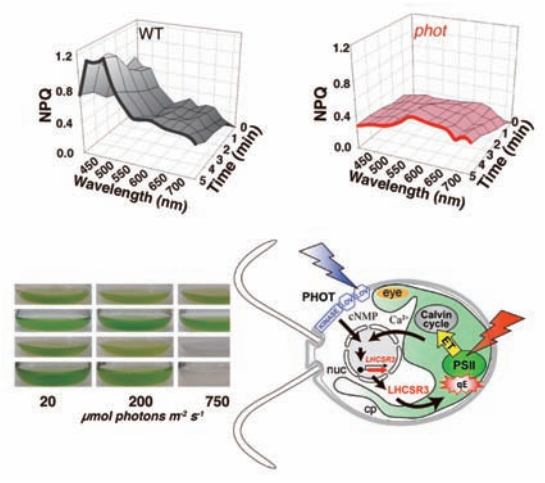


Figure 2. PHOT controls induction of LHCSR3 and qE and is crucial for survival of *C. reinhardtii* in high light. NPQ in WT (A) and *phot* (B) cells after exposure for 4 h to different wavelengths of high light). (C) Erlenmeyer flasks containing WT, *acry*, *phot* and *npq4* cells after 16 h of exposure to light of 20, 200 and 750 µmol photons m^{-2} s^{-1}. (D) Schematic representation of the relationship between photoreception, photosynthesis and photoprotection in *C. reinhardtii*. cp, chloroplast; cNMP, cyclic nucleotide mono phosphate (cAMP or cGMP); ET, electron transport; eye, eyespot; nuc, nucleus; PSII, photosystem II.

II. Photoprotection mechanism in symbiotic algae

Reef-building corals harbor endosymbiotic dinoflagellates of the genus *Symbiodinium* and rely on the energy that the algae generate from photosynthesis for their growth and survival. *Symbiodinium* within corals is a major producer in coral reef ecosystems, which represent one of the most biologically rich environments on earth. When the photosynthesis in *Symbiodinium* is damaged, corals bleach and the sustainability of the reefs is endangered. Photosynthesis in *Symbiodinium* is sensitive to small increases in seawater temperature, resulting in photoinhibition of photosynthesis. The thermal sensitivity of *Symbiodinium* to photoinhibition differs among *Symbiodinium* strains but its mechanism has not been well understood.

Previous studies have demonstrated that increase in seawater temperature enhances cyclic electron flow (CEF), which sustains photoprotective thermal energy dissipation (qE), in *Symbiodinium*. However, the result was still controversial. Furthermore, it was uncertain whether this ability differs among *Symbiodinium* strains. We therefore examined the effect of increased temperature on CEF using different *Symbiodinium* strains. The light-dependent reduction of the primary electron donor PSI, i.e., P700+, was enhanced in all *Symbiodinium* strains by increasing temperatures, indicating CEF was induced by heat, which is accompanied by qE activation. However the critical temperatures for inducing CEF were different among *Symbiodinium* strains. The clade A strains with greater susceptibility to photoinhibition, OTcH-1 and Y106, exhibited higher CEF activities under moderate heat stress than a more phototolerant clade B strain Mf1.05b, suggesting that the observed CEF induction was not a preventive protection mechanism, but was a stress response in *Symbiodinium*.

III. Species specificity in coral-algae symbiosis

Symbiodinium are genetically diverse and their physiological characteristics (e.g., stress sensitivity) differ among phylotypes. Therefore, corals need to recruit *Symbiodinium* phylotypes that suit the environment in order to survive and adapt to changes (e.g., global change and warming). Interestingly, each coral species associate only with specific *Symbiodinium* phylotypes, consequently the diversity of symbionts available differs among coral species. However, the mechanism regulating the diversity of compatible symbionts in cnidarian organisms, including coral, was unknown.

We studied how corals select symbionts and what determines symbiont diversity in each coral species. In our study, we focus on the difference of cell size among *Symbiodinium* strains. Using a model *Aiptasia-Symbiodinium* system, we first found that, of *Symbiodinium* strains tested, only large-sized strains failed to infect the *Aiptasia* host. This size-dependency was supported by experiments using fluorescent microspheres of different sizes. We then tested the uptake of different sized *Symbiodinium* strains into aposymbiotic polyps from two different coral species. *Acropora tenuis* showed the same preference as *Aiptasia*, with no infection by the large-sized *Symbiodinium* strains. However, for *Cyphastrea serailia* all *Symbiodinium* strains tested, including the large-sized strains, were able to infect the host. Our results demonstrated that the infectivity of each *Symbiodinium* strains in a host is primarily determined by their cell size and that the diversity of symbionts in each host species is determined by their maximum acceptable symbiont cell size. We proposed that corals with a higher maximum threshold for symbiont cell size may have the opportunity to associate with more diverse *Symbiodinium* phylotypes. Such coral species may be better able to adapt to changing environmental conditions, and more specifically might be more suited to avoiding bleaching under increasing ocean temperatures.

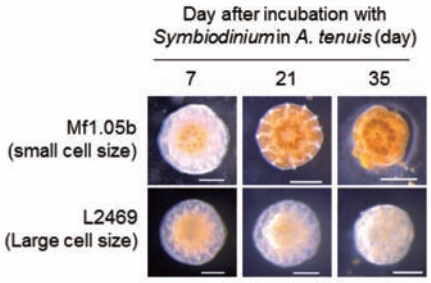


Figure 3. Infection of different Symbiodinium strains into corals. Aposymbiotic primal polyps of *A. tenuis* (scale bars, 500 µm) were separately incubated with small (Mf1.05b) and large (L2469) *Symbiodinium* strains. Uptake of *Symbiodinium* into coral polyps was monitored using a stereomicroscope.

Publication List:

〔Original Papers〕

- Wang, L., Yamano, T., Takane, S., Niikawa, Y., Toyokawa, C., Ozawa, S., Tokutsu, R., Takahashi, Y., Minagawa, J., Kanesaki, Y., Yoshikawa, H., and Fukuzawa, H. (2016). Chloroplast-mediated regulation of CO_2-concentrating mechanism by Ca^{2+}-binding protein CAS in the green

alga *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA *113*, 12586-12591.

- Petroutsos, D.*, Tokutsu, R.*, Maruyama, S., Flori, S., Greiner, A., Magneschi, L., Cusant, L., Kottke, T., Mittag, M., Hegemann, P., Finazzi, G., and Minagawa, J. (2016). A blue light photoreceptor mediates the feedback regulation of photosynthesis. Nature *537*, 563-566. (*: Co-first authors)
- Ueki, N., Ide, T., Mochiji, S., Kobayashi, Y., Tokutsu, R., Ohnishi, N., Yamaguchi, K., Shigenobu, S., Tanaka, K., Minagawa, J., Hisabori, T., Hirono, and M., Wakabayashi, K. (2016). Eyespot-dependent determination of the phototactic sign in *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA *113*, 5299-5304.
- Aihara, Y., Takahashi, S., and Minagawa, J. (2016). Heat induction of cyclic electron flow around photosystem I in the symbiotic dinoflagellate *Symbiodinium*. Plant Physiol. *171*, 522-529.
- Watanabe, C.K., Yamori, W., Takahashi, S., Terashima, I., and Noguchi, K. (2016). Mitochondrial alternative pathway-associated photoprotection of photosystem II is related to the photorespiratory pathway. Plant Cell Physiol. *57*, 1426-1431.
- Yamamoto, H., Takahashi, S., Murray, R.B., and Shikanai, T. (2016). Artificial remodelling of alternative electron flow by flavodiiron proteins in *Arabidopsis*. Nature Plants *2*, 16012.

DIVISION OF SEASONAL BIOLOGY (ADJUNCT)

Adjunct Professor
YOSHIMURA, Takashi

Specially Appointed Assistant Professor: *SHINOMIYA, Ai*
SHIMMURA, Tsuyoshi
Visiting Graduate Student: *NAKAYAMA, Tomoya*
NAKATSUKASA, Mana
SHIMO, Takayuki
ADACHI, Daisuke
Visiting Undergraduate: *MARUYAMA, Michiyo*
Technical Assistant: *AKAMA, Akiko*
KINOSHITA, Chie
BABA, Nayumi
Secretary: *OKUBO, Masayo*

Animals living outside the tropics adapt various physiology and behavior to seasonal changes in the environment. For example, animals restrict breeding to specific seasons to maximize survival of their offspring in temperate zones. As animals use changes in day length and temperature as seasonal cues, these phenomena are referred to as photoperiodism and thermoperiodism, respectively. We use comparative approaches to understand these mechanisms. Medaka fish provides an excellent model to study these mechanisms because of their rapid and robust seasonal responses. In this division, we are trying to uncover the underlying mechanisms of seasonal adaptation.

I. Underlying mechanism that defines the critical photoperiod

It is well established that the circadian clock (i.e., an internal biological clock with a period of approximately 24 hrs) is somehow involved in seasonal time measurement.

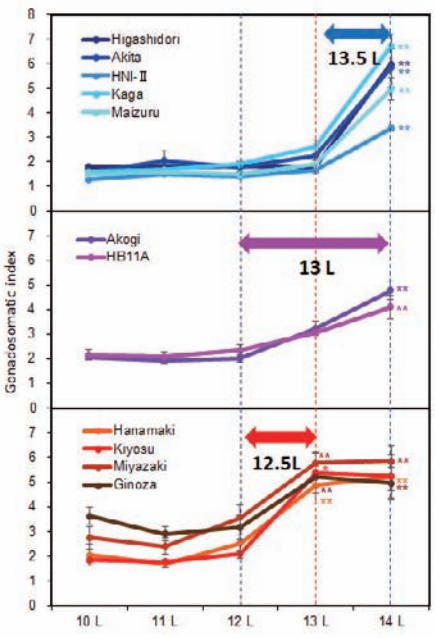


Figure 1. Different critical day length between Medaka from higher and lower latitudes.

However, it remains unknown how the circadian clock measures day length. It has been reported that Medaka populations that were caught at higher latitudes have more sophisticated responses to day length (Sawara and Egami, 1977). For example, Medaka fish caught in Hokkaido have a longer critical day length (i.e., duration of photoperiod required to cause a response) than those caught in Okinawa. To uncover the underlying mechanism of seasonal time measurement, we are currently performing a forward genetic analysis in Medaka populations collected from various latitudes all over Japan.

1-1 Variation in critical photoperiod with latitude in Medaka fish

To perform a forward genetic analysis, we have obtained 11 populations including wild populations, closed colonies, and inbred strains from all over Japan. We have examined the effects of changing day length to determine the critical day lengths that will cause seasonal responses in the gonad. In winter, fish were subjected to 10, 11, 12, 13, and 14 h day lengths with warm temperatures. Then gonadal development was examined to determine the critical day length. As a result, we found differences in the critical day length among Medaka populations. That is, Medaka from higher latitudes required longer day length while those from lower latitudes required shorter day length (Figure 1).

1-2 Quantitative trait loci (QTL) analysis of critical day length

To identify the genes regulating critical day length, quantitative trait loci (QTL) analysis was conducted using F_2 medaka derived from crosses between Northern and Southern populations. As a result, we identified significant QTLs using Restriction-site Associated DNA (RAD) markers (Figure 2). We are now performing whole genome re-sequencing using various Medaka strains that show different critical photoperiods.

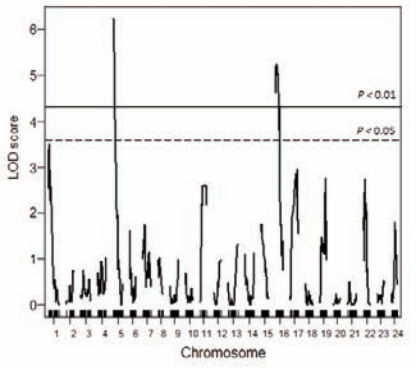


Figure 2. Result of QTL analysis for critical day length.

II. Mechanism that determines seasonal breeders and non-seasonal breeders

Animals that reproduce year-round (e.g., human beings and laboratory mice) are so-called non-seasonal breeders. In contrast most animals living outside of tropical zones reproduce only during a particular period of the year. Therefore, they are called seasonal breeders. However, the underlying

mechanism that determines seasonal breeders and non-seasonal breeders remains unknown. To uncover this mechanism, we performed a forward genetic approach.

2-1 Geographic variations in the responses to short day stimulus

When we transferred Medaka fish from summer conditions to winter conditions, we noticed that Medaka from lower latitudes do not regress their gonads even under short day conditions. Accordingly, we next examined the responses to short day conditions using 19 populations derived from various latitudes. As a result, populations from higher latitudes showed gonadal regression, while populations from lower latitudes did not regress their gonads (Figure 3).

Figure 3. Medaka from lower latitudes (Solid black symbols) do not regress their gonads even under short day conditions.

2-2 QTL analysis of genes determining seasonal breeders and non-seasonal breeders

To identify genes that determine seasonal breeders and non-seasonal breeders, we performed QTL analysis using F_2 generations and identified a significant QTL that determines seasonal breeders and non-seasonal breeders. We are currently trying to identify responsible genes (Figure 4).

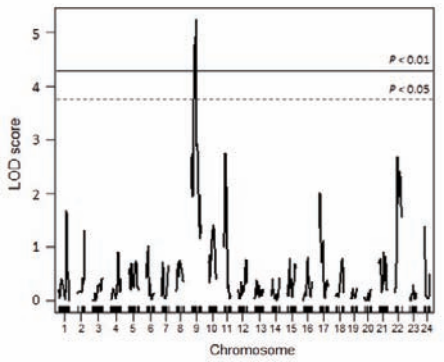


Figure 4. Result of QTL analysis that determines seasonal breeder and non-seasonal breeder.

III. Transcriptome analysis of seasonality in Medaka fish

In addition to the forward genetic approach, we have performed genome-wide transcriptome analysis of brain, eye, and liver of Medaka fish to understand the underlying mechanism of seasonal adaptation. Results of the eyes revealed dynamic seasonal changes in expression of genes encoding photopigments and the downstream phototransduction pathway. Functional analysis suggested that seasonally regulated plasticity in the phototransduction pathway is critical for the emergence of seasonally regulated behavior.

Publication List:

〔Review articles〕

- Ikegami, K., and Yoshimura, T. (2016). Comparative analysis reveals the underlying mechanism of vertebrate seasonal reproduction. Gen. Comp. Endocrinol. *227*, 64-68.
- Nishiwaki-Ohkawa, T., and Yoshimura, T. (2016). Molecular basis for regulating seasonal reproduction in vertebrates. J. Endocrinol. *229*, R117-R127.

LABORATORY OF GENOME INFORMATICS

Assistant Professor
UCHIYAMA, Ikuo

Postdoctoral Fellow: *CHIBA, Hirokazu*

The accumulation of biological data has recently been accelerated by various high-throughput "omics" technologies including genomics, transcriptomics, and proteomics. The field of genome informatics is aimed at utilizing this data, or finding the principles behind the data, for understanding complex living systems by integrating the data with current biological knowledge using various computational techniques. In this laboratory we focus on developing computational methods and tools for comparative genome analysis, which is a useful approach for finding functional or evolutionary clues to interpreting the genomic information of various species. The current focus of our research is on the comparative analysis of microbial genomes, the number of which has been increasing rapidly. Since different types of information about biological functions and evolutionary processes can be extracted from comparisons of genomes at different evolutionary distances, we are developing methods to conduct comparative analyses not only of distantly related but also of closely related genomes.

I. Microbial genome database for comparative analysis

The microbial genome database for comparative analysis (MBGD; URL http://mbgd.genome.ad.jp/) is a comprehensive platform for microbial comparative genomics. The central function of MBGD is to create orthologous groups among multiple genomes using the DomClust program combined with the DomRefine program (see Section II below). By means of these programs, MBGD not only provides comprehensive orthologous groups among the latest genomic data, but also allows users to create their own ortholog groups on the fly using a specified set of organisms. MBGD also has pre-calculated ortholog tables for each major taxonomic group, and provides several views to display the entire picture of each ortholog table. For some closely related taxa, MBGD provides the conserved synteny information calculated using the CoreAligner program. In addition, MBGD provides MyMBGD mode, which allows users to add their own genomes to MBGD. Moreover, MBGD now stores recently accumulating draft genome data, and allows users to incorporate them into a user specific ortholog database through the MyMBGD functionality.

To cope with the rapid growth of microbial genome data, we are trying to establish an efficient protocol to maintain the ortholog tables. We are now developing a progressive strategy to create ortholog data: it first creates an intra-species ortholog table and generates a species pan-genome; next it creates an intra-genera ortholog table and generates a genus pan-genome; and finally it conducts an inter-genera comparison to create an ortholog table covering the entire taxonomic range. This strategy can integrate the multiple ortholog tables currently created: the standard ortholog table created from representative species and the taxon-specific ortholog tables for major taxa. The strategy can also reduce the computation time to calculate all-against-all similarities because it calculates accurate similarities only for inter-genera comparisons and uses a much more rapid (but less accurate) program to calculate intra-species and intra-genus similarities.

II. Orthologous gene classification among multiple genomes at the domain level

As a core technology of our comparative genomics tools, we developed a rapid automated method of ortholog grouping, named DomClust, which is effective enough to allow the comparison of many genomes simultaneously. The method classifies genes based on a hierarchical clustering algorithm using all-against-all similarity data as an input, but it also detects domain fusion or fission events and splits clusters into domains if required. As a result, the procedure can split genes into the domains minimally required for ortholog grouping.

We also developed a procedure to refine the DomClust classification based on multiple sequence alignments instead of pairwise sequence alignments. The method is based on a scoring scheme, the domain-specific sum-of-pairs (DSP) score, which evaluates domain-level classification using the sum total of domain-level alignment scores. We developed a refinement pipeline to improve domain-level clustering, DomRefine, by optimizing the DSP score. DomRefine is now used to construct the standard ortholog table covering all the representative genomes stored in MBGD.

Domain-level classification is a unique feature of our ortholog classification system. In fact, it is different from conventional domain databases like Pfam in that it is based on orthology instead of homology. Particularly, this data is considered to be suitable for analyzing domain fusion events that occurred during evolution. Now, by analyzing the domain-level ortholog grouping data combined with taxonomic and functional information, we are trying to elucidate when and in what kind of genes domain fusion events frequently occurred.

III. Development of a workbench for comparative genomics

We have developed a comparative genomics workbench named RECOG (Research Environment for COmparative Genomics), which aims to extend the current MBGD system by incorporating more advanced analysis functionalities. The central function of RECOG is to display and manipulate a large-scale ortholog table. The ortholog table viewer is a spreadsheet like viewer that can display the entire ortholog table, containing more than a thousand genomes. In RECOG, several basic operations on the ortholog table are defined, which are classified into categories such as filtering, sorting, and coloring, and various comparative analyses can be done by combining these basic operations. In addition, RECOG allows the user to input arbitrary gene properties and compare these properties among orthologs in various genomes.

We continue to develop the system and apply it to various genome comparison studies under collaborative research projects (including *H. pylori* genome comparison described in Section V below). In particular, in addition to microbial genome comparison, we are trying to apply RECOG to comparative analyses of transcriptomic data and metagenomic data.

IV. Ortholog data representation using the Semantic Web technology to integrate various microbial databases

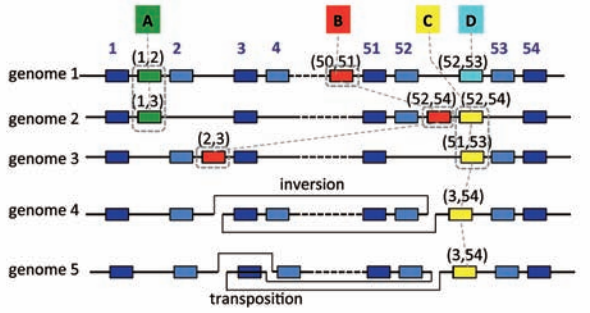
Orthology is a key to integrate knowledge about various organisms through comparative analysis. We have constructed an ortholog database using Semantic Web technology, aiming at the integration of numerous genomic data and various types of biological information. To formalize the structure of the ortholog information in the Semantic Web, we developed an ortholog ontology (OrthO) and described the ortholog information in MBGD in the form of the Resource Description Framework (RDF). On the basis of this framework, we have integrated various kinds of microbial data using the ortholog information as a hub, as part of the MicrobeDB.jp project under the National Bioscience Database Center.

This year, to further standardize the ontology, we developed the Orthology Ontology (ORTH) in collaboration with Dr. Fernandez-Breis (Univ.Murcia) by integrating OrthO and OGO (another ortholog ontology developed by Dr. Fernandez-Breis) and reusing other existing ontologies.

In addition, to facilitate the utilization of the RDF databases distributed worldwide, we developed a command-line tool, named SPANG. SPANG simplifies querying distributed RDF stores using the SPARQL query language, and provides a framework for reusing and sharing queries across the Web, thereby reducing the burden of writing complex queries in SPARQL.

V. *H. pylori* pan-genome analysis for identification of genomic islands

Genomes of bacterial species can show great variation in their gene content, and thus systematic analysis of the entire gene repertoire, termed the "pan-genome", is important for understanding bacterial intra-species diversity. We analyzed the pan-genome identified among 30 strains of the human gastric pathogen *Helicobacter pylori* isolated from various phylogeographical groups. We developed a method (FindMobile) to define mobility of genes against the reference coordinate determined by the core alignment created by CoreAligner, and classified each non-core gene into mobility classes (Figure 1). In addition, by clustering the accessory OGs on the basis of phylogenetic pattern similarity and chromosomal proximity, we identified 60 co-occurring gene clusters (CGCs). Besides known genomic islands including *cag* pathogenicity island, bacteriophages, and integrating conjugative elements, we identified some novel ones, including TerY-phosphorylation triad and that containing a reverse-transcriptase homolog.



OG	mobility extent	non-consecutive	number of genes	N_g	class
A	1	0	2	2	Stable
B	3	0	3	3	Mobile
C	1	2	4	2	Intermediate
D	1	0	1	1	Unique

Figure 1. Definition of the mobility classes in FindMobile. Boxes in dark blue and pale blue represent fully conserved and partially conserved core genes, respectively. Boxes in the other colors are non-core genes that are here classified into four classes: A) stable, B) mobile, C) intermediate, D) unique.

Publication List:

〔Original papers〕

- Fernández-Breis, J.T. Chiba, H., Legaz-García, M.C., and Uchiyama, I. (2016). The orthology ontology: development and applications. J. Biomed. Semant. *7*, 34.
- Hoshino, A., Jayakumar, V., Nitasaka, E., Toyoda, A., Noguchi, H., Itoh, T., Shin-I, T., Minakuchi, Y., Koda, Y., Nagano, A.J., Yasugi, M., Honjo, M.N., Kudoh, H., Seki, M., Kamiya, A., Shiraki, T., Carninci, P., Asamizu, E., Nishide, H., Tanaka, S., Park, K., Morita, Y., Yokoyama, K., Uchiyama, I., Tanaka, Y., Tabata, S., Shinozaki, K., Hayashizaki, Y., Kohara, Y., Suzuki, Y., Sugano, S., Fujiyama, A., Iida, S., and Sakakibara, Y. (2016). Genome sequence and analysis of the Japanese morning glory *Ipomoea nil*. Nat. Commun. *7*, 13295.
- Matsui, H., Takahashi, T., Murayama, S., Uchiyama, I., Yamaguchi, K., Shigenobu, S., Suzuki, M., Rimbara, E., Shibayama, K., Overby, A., and Nakamura, M. (2016). Draft genome sequence of *Helicobacter suis* strain SNTW101, isolated from a Japanese patient with nodular gastritis. Genome Announc. *5*, e00934-16.
- Nakai, R., Fujisawa, T., Nakamura, Y., Nishide, H., Uchiyama, I., Baba, T., Toyoda, A., Fujiyama, A., Naganuma, T., and Niki, H. (2016). Complete genome sequence of *Aurantimicrobium minutum* type strain KNCT, a planktonic ultramicrobacterium isolated from river water. Genome Announc. *4*, e00616.
- Uchiyama, I., Albritton, J., Fukuyo, M., Kojima, K., Yahara, K., and Kobayashi, I. (2016). A novel approach to *Helicobacter pylori* pan-genome analysis for identification of genomic islands. PLoS One *11*, e0159419.
- Yahara, K., Furuta, Y., Morimoto, S., Kikutake, C., Komukai, S., Matelska, D., Dunin-Horkawicz, S., Bujnicki, J.M., Uchiyama, I., and Kobayashi, I. (2016). Genome-wide survey of codons under diversifying selection in a highly recombining bacterial species, *Helicobacter pylori*. DNA Res. *23*, 135-143.

LABORATORY FOR SPATIOTEMPORAL REGULATIONS

Associate Professor
NONAKA, Shigenori

NIBB Research Fellow: *TANIGUCHI, Atsushi**
Postdoctoral Fellow: *TANIGUCHI, Atsushi*
Technical Assistant: *ISHIBASHI, Tomoko*

Our laboratory is currently pursuing two paths of scientific inquiry that are of interest to developmental biology. One goal is to clarify the mechanism of left-right (L-R) determination. The other is to develop imaging technologies for biological analyses.

I. Initial step for left-right asymmetry

In mammalian development, the initial left-right asymmetry first appears as cilia-driven leftward fluid flow on the ventral surface of the node, and the flow provides the initial cue for asymmetric gene expressions that actually determine asymmetric morphogenesis. The mechanisms that convert the flow to asymmetric gene expression, i.e. the flow sensing mechanism, remains controversial, with several models being proposed, and involvement of Ca^{2+} being suggested.

We pursued this question by measuring Ca^{2+} dynamics in the node and found that the node cells cause apparently stochastic elevation of Ca^{2+}, and its spatiotemporal distribution is equal at the left and right sides but becomes more frequent at the left after late headfold stage, when the flow-induced commitment occurs. This asymmetry was disrupted in dynein mutant *iv*/*iv* and *pkd2*$^{-/-}$ mutants, in accordance to their left-right phenotypes.

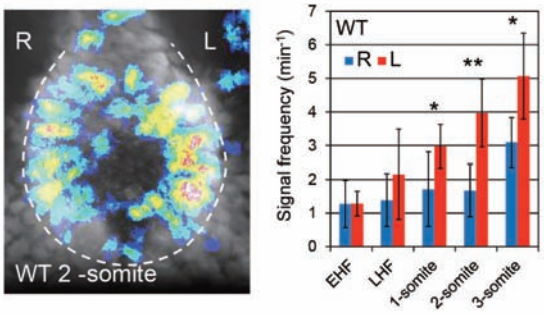


Figure 1. Left: Distribution of Ca^{2+} elevation in a 2-somite wild-type node. Right: Time course of Ca^{2+} elevation frequency at the left and the right sides.

Based on these findings, we are now examining the validity of the two major proposed models of flow sensing, mechanosensing and chemosensing, and a third novel mechanism.

II. Development of light-sheet microscopy

Light-sheet microscopy has become popular over this decade for its advantages including low photodamage and fast image acquisition. We are now running two light-sheet microscopes, one commercial and the other self-made, maintaining them for both collaborations and for our own research interest, left-right asymmetry.

Over several years we have developed a fast light-sheet microscope named ezDSLM, in which the excitation light sheet and detection objective move instead of the specimen for z-scanning. We installed an electrically tunable lens (ETL) to achieve further speed and exclusion of mechanical vibrations that disturb continuous 4D imaging of floating or loosely held specimens.

Our light-sheet microscopes are open to other researchers via NIBB's Collaborative Research and MEXT's Advanced Bioimaging Support (ABiS) programs. Numerous collaborations are in progress including observation of moving *Amoeba proteus*, neuronal activity in *Drosophila* larvae, cell migration in zebrafish embryos, cleared mouse brains, and marine crustaceans, etc.

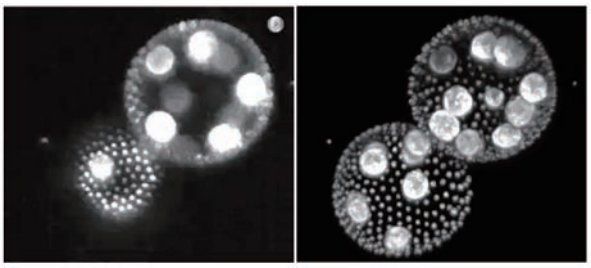


Figure 2. Images of floating volvox taken by ezDSLM with ETL. Left: Single optical section. Right: Maximum intensity projection.

LABORATORY OF NUCLEAR DYNAMICS

Specially Appointed Associate Professor
MIYANARI, Yusuke

Postdoctoral Fellow: *KURIHARA, Misuzu*
SOKENDAI Graduate Student: *ISHII, Satoko*
Visiting Graduate Student: *KAKIZUKA, Taishi*
Technical Assistant: *SANBO, Chiaki*
TAGAWA, Ayako
Secretary: *HACHISUKA, Midori*

A fundamental question in biology is to understand the mechanisms underlying cell-fate decision. Genomic reprogramming after mammalian fertilization reverts terminally differentiated gametes into toti- or pluri-potent states to start a new developmental program. Cell lineage allocation in the reprogramming process is accompanied by drastic changes in the pattern of gene expression, epigenetic configurations, and nuclear organization. We aim to reveal the roles of chromatin dynamics in cell lineage-allocation by deciphering the molecular mechanisms underlying remodeling of nuclear organization and their effects on developmental gene expression, using mouse embryos and embryonic stem (ES) cells as model systems.

Epigenetic reprogramming in early mouse embryos.

Genomic reprogramming reverts fully differentiated cells to a totipotent state to start a new developmental program. In the early mouse embryo, terminally differentiated gametes are reprogrammed after fertilization thereby acquiring a totipotent state. Upon the fourth cleavage, 8-cell stage embryos, which have undergone the process of compaction, will give rise to the morula (Figure 1). The outer cells of the morula will differentiate into the epithelial trophectoderm (TE) of the blastocyst. The inner cells of the morula will become the inner cell mass (ICM) of the blastocyst. The ICM subsequently leads to the formation of two lineages, epiblast (EPI) and primitive endoderm (PE), with the former representing pluripotency as it gives rise to the embryo itself. The reprogramming event is accompanied by epigenetic modifications and changes in chromatin structures throughout the embryo, which are essential for regulation of gene expression involved in differentiation, and reprogramming of the EPI.

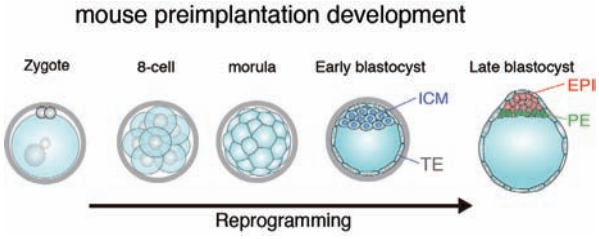


Figure 1. Lineage allocation in mouse preimplantation development

Remodeling of nuclear architecture in development

Chromatin is organized in a non-random fashion within three-dimensional nuclear space. During developmental processes, nuclear architecture is dramatically reconstructed, resulting in establishment of cell-type specific nuclear organization. Defects in structural components of the nucleus are responsible for developmental aberrations and several human diseases. Remodeling of nuclear architecture leads to spatial arrangement of genes, which could affect genome functions including gene expression. However, regulatory mechanisms underlying nuclear reorganization during cell-fate decision remains largely unknown.

Chromatin structure

Spatiotemporal organization of genomic DNA within the nucleus is suggested as an emerging key player to regulate gene expression. The developmental program accompanies nuclear remodeling, resulting in construction of cell-type specific nuclear architecture. Firstly, chromosomes are confined in discrete nuclear spaces, "chromosome territories" (Figure 2). Within them, further levels of 3D organization, "topologically associating domains" (TADs), are observed. TADs can be defined as linear units of chromatin containing several gene loci, and fold as discrete 3D structures in which gene loci frequently interact with each other. Recent works have revealed that folding of "local" chromatin structures such as enhancer-promoter looping is associated with genome functions. Despite the drastic changes of these hierarchical chromatin structures, their role in cell-fate decision remains largely unexplored.

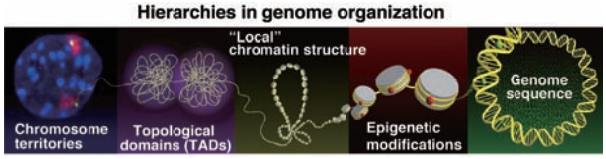


Figure 2. Hierarchical chromatin structure

Approach

We have developed a powerful imaging technology termed TALE-mediated Genome Visualization (TGV), which allows us to track specific genomic sequences in living cells (Miyanari Y, Nature Structural & Molecular Biology, 2013). Importantly, this technique is versatile and can be extended to allow many robust applications, which will be integrated into our study to manipulate several genome functions. Based on new technological development, we aim to understand biological roles of chromatin dynamics in cell-fate decision.

Publication List:

〔Review article〕

● Miyanari, Y. (2016). TAL effector-mediated Genome Visualization (TGV). Methods *69*. 198-204.

LABORATORY OF PLANT DEVELOPMENT AND PHYSIOLOGY

Specially Appointed Associate Professor
KAWADE, Kensuke

Adjunct Professor (BIO-NEXT Project, OIIB): *TSUKAYA, Hirokazu*
Postdoctoral Fellow: *NOZAKI, Mamoru*
Visiting Graduate Student: *TOMOI, Takumi*
Technical Assistant: *YAMAGUCHI, Chinami*
Secretary: *HACHISUKA, Midori*

There has been growing evidence that metabolic regulation has specific impacts on plant development. The picture emerging depicts the metabolism as a dynamic system that controls and/or supports developmental progression. Despite these advances, it remains largely unclear how metabolism is regulated behind developmental process. We aim to uncover as-yet-unknown relationships between developmental and metabolic processes in plants and their biological meaning by elucidating molecular mechanisms for the system. To address this, we primary use trans-omics approach including metabolome and transcriptome analyses using *Arabidopsis thaliana* as a model, in conjunction with standard molecular genetics and biochemistry techniques.

I. Functional screening of orphan metabolic enzymes from phenome data

To explore as-yet-unknown relationships between developmental and metabolic processes, we carried out functional screening of *Arabidopsis thaliana* mutants of orphan genes encoding metabolic enzymes. We examined 12 non-biased traits including leaf size, primary root length, seed color, *etc*. A large number of mutants grew normally compared to wild type (WT), probably due to gene functional redundancy. However, we found that cotyledon size is smaller and more variable in one mutant line. A more severe morphological defect in this mutant is irregular arrangement of cotyledons. Although bilateral arrangement of cotyledons was observed in WT, this mutant forms non-bilateral, single or cup-shaped cotyledon (Figure 1). This defect in cotyledon morphology/arrangement could be detected from early embryo development. Confocal microscopy using various auxin-related markers revealed that cellular polarity is often disordered by this mutation, resulted in abnormal distribution of auxin in the mutant embryos. To identify the *in vivo* metabolic target of this enzyme, metabolome analysis using gas-chromatography mass-spectrometry (GC-MS) will be performed.

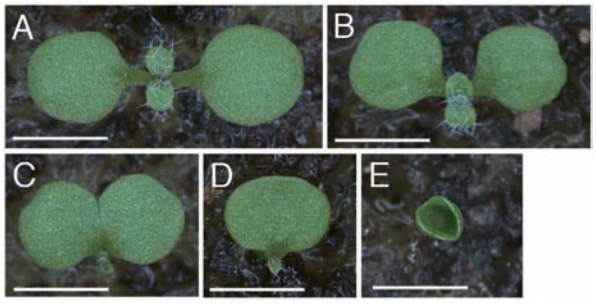


Figure 1. Irregular arrangement of cotyledons in the enzyme mutant. Wild type has two bilateral cotyledons (A). In contrast, our enzyme mutant shows abnormally-arranged (B), fused (C), single (D) and cup-shaped cotyledons (E). Bars = 2 mm.

II. Characterization of developmental signal intertwined with metabolism control

We recently uncovered that one of the plant developmental signals forms an expression gradient along the leaf proximal-to-distal axis to determine the cell-proliferation domain (Kawade et al., *submitted*). By metabolome and transcriptome analyses, we have uncovered that this developmental signal regulates not only cell proliferation but also leucine metabolism and/or tricarboxylic acid (TCA) cycle (Figure 2). When we cultured an *Arabidopsis thaliana* mutant of this developmental signal on culture media with higher leucine, this mutant showed hypersensitivity to this amino acid, resulted in growth arrest. Interestingly, we found that redox condition in this mutant was perturbed at the transcriptome level. We are now re-confirming the change in metabolic profile using liquid chromatography-mass spectrometry (LC-MS) analysis in collaboration with the Functional Genomics Facility in NIBB, and also examining the mutant's redox status to clarify an interaction of leucine metabolism and/or TCA cycle with redox homeostasis. This study would provide new insights into how a developmental signal coordinately regulates primary metabolism and redox condition, and then how this metabolic homeostasis has functional importance on developmental processes including cell proliferation.

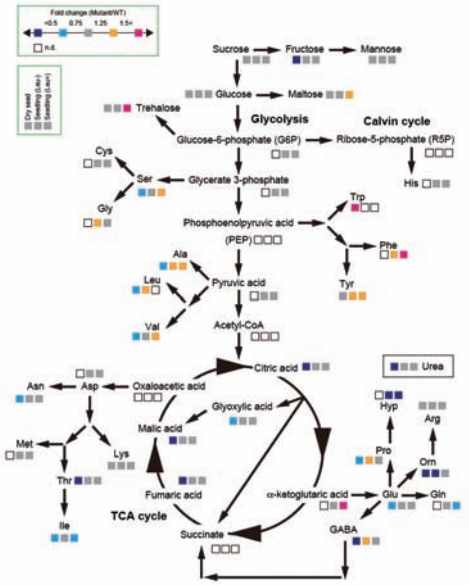


Figure 2. Metabolic change in the mutant of the developmental signaling detected by GC-MS metabolomics technique. Colors indicate change in metabolite content in dry seed, normally cultured seedling and seedling treated with leucine (left to right). Sugars, amino acids and organic acids in the TCA cycle are mainly summarized here.

NIBB Core Research Facilities

Head
YOSHIDA, Shosei

The NIBB Core Research Facilities were launched in 2010 to support basic biology research in NIBB. They consist of three facilities that are developing and providing state-of-the-art technologies to understand biological functions through functional genomics, bioimaging, and bioinformatics.

The NIBB Core Research Facilities also act as an intellectual hub to promote collaboration among the researchers of NIBB and other academic institutions.

Functional Genomics Facility

Specially Appointed Associate Professor:
SHIGENOBU, Shuji

Technical Staff:	*MORI, Tomoko*
	MAKINO, Yumiko
	YAMAGUCHI, Katsushi
	BINO, Takahiro
Technical Assistant:	*ASAO, Hisayo*
	AKITA, Asaka
	MATSUMOTO, Miwako
Secretary:	*ICHIKAWA, Mariko*

The Functional Genomics Facility is a division of the NIBB Core Research Facilities and is organized jointly by NIBB and NIPS for promoting DNA and protein studies. The facility maintains a wide array of core research equipment, from standard machinery like ultracentrifuges to cutting edge tools such as next generation DNA sequencers, which amount to 60 different kinds of instruments. The facility is dedicated to fostering collaborations with researchers both of NIBB and other academic institutions worldwide by providing these tools as well as expertise. Our current focus is supporting functional genomics studies that utilize mass spectrometers and DNA sequencers. We also act as a bridge between experimental biology and bioinformatics.

We recently largely renovated the building of the Functional Genomics Facility. For example, the Visitors Lab and the Visitors Office were newly designed so that we can promote collaboration projects. Indeed, in 2016, more than 200 researchers visited to use our new facility and developed active collaborations.

Representative Instruments

Genomics

The advent of next-generation sequencing (NGS) technologies is transforming today's biology by ultra-high-throughput DNA sequencing. Utilizing HiSeq2500, HiSeq1500, and MiSeq (Illumina), and PacBio RS II (PacificBio Sciences), the Functional Genomics Facility is committed to joint research aiming to explore otherwise inaccessible new fields in basic biology.

During 2016 we carried out 52 NGS projects in collaboration with NIBB laboratories as well as the researchers of other academic institutions. These projects cover a wide range of species (bacteria, animals, plants, and humans) including both model and non-model organisms, and various applications such as genomic re-sequencing, RNA-seq and ChIP-seq.

Figure 1. Next-generation sequencer

Proteomics

Three different types of mass spectrometer and two protein sequencers, as listed below, are used for proteome studies in our facility. In 2016, we analyzed approximately 700 samples with mass spectrometers and protein sequencers.

- LC-MS (AB SCIEX TripleTOF 5600 system)
- LC-MS (Thermo Fisher SCIENTIFIC Orbitrap Elite)
- MALDI-TOF-MS (Bruker Daltonics REFLEX III)
- LC-Q-TOF MS (Waters Q-TOF Premier)
- Protein sequencer (ABI Procise 494 HT; ABI Procise 492 cLC)

Other analytical instruments (excerpts)

- Cell sorter (SONY SH800, BD FACS Aria II)
- Bioimaging Analyzer (Fujifilm LAS 3000 mini; GE FLA9000)
- Laser Capture Microdissection System (Arcturus XT)
- DNA Sequencer (ABI 3130xl)
- Real Time PCR (ABI 7500)
- Ultra Centrifuge (Beckman XL-80XP etc.)

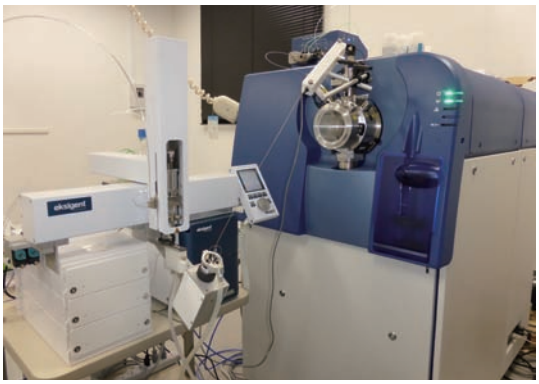


Figure 2. Triple TOF LC/MS/MS System

Genome Informatics Training Course

We organize NIBB Genome Informatics Training Courses every year. These courses are designed to introduce basic knowledge and skills of bioinformatics to biologists who are not familiar with bioinformatics. In 2016, we provided two courses on RNA-seq data analysis and on BLAST sequence analysis, where a total of 107 researchers and students joined from all over Japan.

Figure 3. NIBB Genome Informatics Training Course

Publication List on Cooperation:

〔Original papers〕

- Akashi, H.D., Cádiz Díaz, A., Shigenobu, S., Makino, T., and Kawata, M. (2016). Differentially expressed genes associated with adaptation to different thermal environments in three sympatric cuban anolis lizards. Molec. Ecol. *25*, 2273–2285.
- Higo, A., Niwa, M., Yamato, K.T., Yamada, L., Sawada, H., Sakamoto, T., Kurata, T., Shirakawa, M., Endo, M., Shigenobu, S., *et al*. (2016). Transcriptional framework of male gametogenesis in the liverwort *Marchantia polymorpha* L. Plant Cell Physiol. *57*, 325–338.
- Ide, T., Mochiji, S., Ueki, N., Yamaguchi, K., Shigenobu, S., Hirono, M., and Wakabayashi, K. (2016). Identification of the *agg1* mutation responsible for negative phototaxis in a "wild-type" strain of *Chlamydomonas reinhardtii*. Biochem. Biophys. Rep. *7*, 379-385.
- Koga, H., Fujitani, H., Morino, Y., Miyamoto, N., Tsuchimoto, J., Shibata, T.F., Nozawa, M., Shigenobu, S., Ogura, A., Tachibana, K., *et al*. (2016). Experimental approach reveals the role of *alx1* in the evolution of the echinoderm larval skeleton. PLoS ONE *11*, e0149067.
- Matsui, H., Takahashi, T., Murayama, S.A., Uchiyama, I., Yamaguchi, K., Shigenobu, S., Suzuki, M., Rimbara, E., Shibayama, K., Øverby, A., and Nakamura, M. (2016). Draft genome sequence of *Helicobacter suis* strain SNTW101, isolated from a Japanese patient with nodular gastritis. Genome Announc. *4*, e00934-16.
- Oda, K., Kamiya, T., Shikanai, Y., Shigenobu, S., Yamaguchi, K., and Fujiwara, T. (2016). The arabidopsis Mg transporter, MRS2-4, is essential for Mg homeostasis under both low and high Mg conditions. Plant Cell Physiol. *57*, 754-763.
- Sato, K., Tanaka, T., Shigenobu, S., Motoi, Y., Wu, J., and Itoh, T. (2016). Improvement of barley genome annotations by deciphering the Haruna Nijo genome. DNA Res. *23*, 21–28.
- Tong, W., Imai, A., Tabata, R., Shigenobu, S., Yamaguchi, K., Yamada, M., Hasebe, M., Sawa, S., Motose, H., and Takahashi, T. (2016). Polyamine resistance is increased by mutations in a nitrate transporter gene NRT1.3 (AtNPF6.4) in *Arabidopsis thaliana*. Front. Plant Sci. *7*, 834.
- Ueki, N., Ide, T., Mochiji, S., Kobayashi, Y., Tokutsu, R., Ohnishi, N., Yamaguchi, K., Shigenobu, S., Tanaka, K., Minagawa, J., Hisabori, T., Hirono, M., and Wakabayashi, K. (2016). Eyespot-dependent determination of the phototactic sign in *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA *113*, 5299-5304.
- Yatsu, R., Miyagawa, S., Kohno, S., Parrott, B.B., Yamaguchi, K., Ogino, Y., Miyakawa, H., Lowers, R.H., Shigenobu, S., Guillette, L.J. Jr., and Iguchi, T. (2016). RNA-seq analysis of the gonadal transcriptome during *Alligator mississippiensis* temperature-dependent sex determination and differentiation. BMC Genomics *17*, 77

〔Original paper (E-publication ahead of print)〕

- Kondo, S., Wakae, K., Wakisaka, N., Nakanishi, Y., Ishikawa, K., Komori, T., Moriyama-Kita, M., Endo, K., Murono, S., Wang, Z., Kitamura, K., Nishiyama, T., Yamaguchi, K., Shigenobu, S., Muramatsu, M., and Yoshizaki, T. APOBEC3A associates with human papillomavirus genome integration in oropharyngeal cancers. Oncogene 2016 Oct 3.
- Murase, K., Shigenobu, S., Fujii, S., Ueda, K., Murata, T., Sakamoto, A., Wada, Y., Yamaguchi, K., Osakabe, Y., Osakabe, K., *et al*. MYB transcription factor gene involved in sex determination in *Asparagus officinalis*. Genes Cells 2016 Nov 21.

● *Research activity by S. Shigenobu*

Specially Appointed Associate Professor: *SHIGENOBU, Shuji*
NIBB Research Fellow: *OGAWA, Kota*
Visiting Graduate Student: *HSIAO, Yi-Min*
Technical Assistant: *SUZUKI, Miyuzu*

Symbiogenomics

"Nothing, it seems, exists except as part of a network of interactions." (Gilbert & Epel, 2008)

Every creature on the earth exists among a network of various biological interactions. For example, many multicellular organisms, including humans, harbor symbiotic bacteria in their bodies: some of them provide their hosts with essential nutrients deficient in the host's diet and others digest foods indigestible by the host alone. In spite of numerous examples of symbioses and its intriguing outcomes, the genetic and molecular basis underlying these interactions remains elusive. The goal of our group is to establish a new interdisciplinary science "Symbiogenomics", where we aim to understand the network of biological interactions at the molecular and genetic level. To this end, we take advantage of state-of-the-art genomics such as next-generation sequencing technologies.

I. Genomic revelations of a mutualism: the pea aphid and its obligate bacterial symbiont

Aphid species bear intracellular symbiotic bacteria in the cytoplasm of bacteriocytes, specialized cells for harboring the bacteria. The mutualism is so obligate that neither can reproduce independently. The 464 Mb draft genome sequence of the pea aphid, *Acyrthosiphon pisum*, in consort with that of bacterial symbiont *Buchnera aphidicola* illustrates the remarkable interdependency between the two organisms. Genetic capacities of the pea aphid and the symbiont for amino acid biosynthesis are complementary. The genome analysis revealed that the pea aphid has undergone characteristic gene losses and duplications. The IMB

antibacterial immune pathway is missing several critical genes, which might account for the evolutionary success of aphids to obtain beneficial symbionts. Lineage-specific gene duplications have occurred in genes in a broad range of functional categories, which include signaling pathways, miRNA machinery, chromatin modification and mitosis. The importance of these duplications for symbiosis remains to be determined. We found several instances of lateral gene transfer from bacteria to the pea aphid genome. Some of them are highly expressed in bacteriocytes.

We recently discovered a novel class of genes in the pea aphid genome that encode small cysteine-rich proteins with secretion signals that are expressed exclusively in bacteriocytes of the pea aphid, and named these bacteriocyte-specific cysteine-rich proteins (BCR). The BCR mRNAs are first expressed at a developmental time point coincident with the incorporation of symbionts strictly in the cells that contribute to the bacteriocyte, and this bacteriocyte-specific expression is maintained throughout the aphid's life. Some BCRs showed an antibiotic activity. These results suggest that BCRs act within bacteriocytes to mediate the symbiosis with bacterial symbionts, which is reminiscent of the cysteine-rich secreted proteins of leguminous plants that also regulate endosymbionts. Employment of small cysteine-rich peptides may be a common tactic of host eukaryotes to manipulate bacterial symbionts.

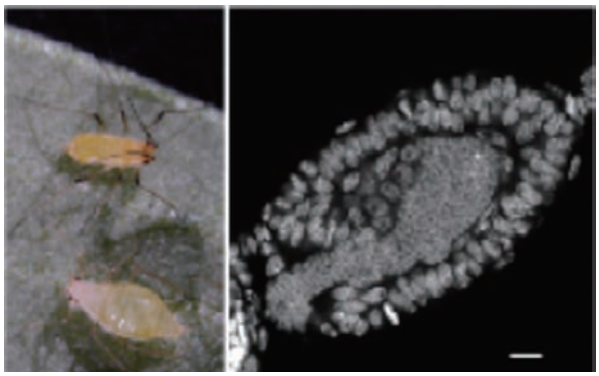


Figure 1. Pea aphids and the bacterial symbiont, *Buchnera*. Adult aphids (Left). A developing viviparous embryo which symbionts are infecting (Right). Scale bar = 20um.

Publication List:

〔Original paper〕

● Hongo, Y., Ikuta, T., Takaki, Y., Shimamura, S., Shigenobu, S., Maruyama, T., and Yoshida, T. (2016). Expression of genes involved in the uptake of inorganic carbon in the gill of a deep-sea vesicomyid clam harboring intracellular thioautotrophic bacteria. Gene *585*, 228-240.

SPECTROGRAPHY AND BIOIMAGING FACILITY

Specially Appointed Associate Professor:
KAMEI, Yasuhiro

Technical Staff:	*KONDO, Maki*
	TANIGUCHI-SAIDA, Misako
	UCHIKAWA, Tamaki
Technical Assistant:	*ICHIKAWA, Chiaki*
	ISHIKAWA, Azusa

The Spectrography and Bioimaging Facility assists both collaborative and core research by managing and maintaining research tools that use "Light". The facility also provides technical support through management of technical staff assisting in the advancement of collaborative and core research projects, as well as academic support to researchers by Dr. Y. Kamei (refer to the Collaborative Research Group Research Enhancement Strategy Office section). Among its tools are advanced microscopes for biology and the Okazaki Large Spectrograph for photobiology. The Okazaki Large Spectrograph is the world's largest wide spectrum exposure mechanism, capable of producing a range of wavelengths from 250 nm (ultraviolet) to 1,000 nm (infrared) along its 10 meter focal curve; allowing exposure to strong monochromatic light. The facility's microscopes, which are cutting edge devices such as confocal and multi-photon excitation microscopes, are used by both internal and external researchers as vital equipment for core and collaborative research projects.

Representative Instruments: Okazaki Large Spectrograph (OLS)

The spectrograph runs on a 30 kW Xenon arc lamp and projects a wavelength spectrum from 250 nm (ultraviolet) to 1,000 nm (infrared) onto its 10 m focal curve with an intensity of monochromatic light at each wavelength more than twice as much as that of the corresponding monochromatic component of tropical sunlight at noon (Watanabe *et al*., Photochem. Photobiol. *36*, 491-498, 1982). The spectrograph is dedicated to action spectroscopical studies of various light-controlled biological processes.

The NIBB Collaborative Research Program for the Use of the OLS supports about 10 projects every year conducted by both visiting scientists, including foreign researchers, as well as those in NIBB.

Action spectroscopical studies for various regulatory and damaging effects of light on living organisms, biological molecules, and artificial organic molecules have been conducted.

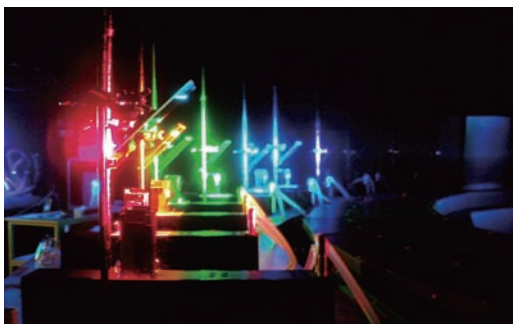


Figure 1. An example of experiments using the Large Spectrograph. Various color rays (monochromatic light from right side and reflected by mirrors) were irradiated simultaneously to samples in cooling chambers.

Microscopes

This facility also has Bioimaging machines such as wide-field microscopes (Olympus IX-81 and BX-63), confocal microscopes (Olympus FV1000, Nikon A1R, Nikon A1Rsi and Yokogawa CSU-X1 with EM-CCD camera), multi-photon microscopes (Olympus FV1000-MP, FV1200-MPs, Leica TCS-SP8 MPs) and other advanced custom-made laser microscopes with special aims (Digital Scanned Light-sheet Microscope: DSLM and Infrared Laser-Evoked Gene Operator microscope: IR-LEGO) for users in NIBB and collaborative guest researchers. We began two new types of Collaborative Research Program. One is a new category of the NIBB Collaborative Research for Integrative Bioimaging using machines and bioimage processing/analysis techniques, and the other is the Advanced Bioimaging Support Program (ABiS) of the Grant-in-aid for Scientific Research on Innovative Areas.

The DSLM was developed by Dr. Ernst Stelzer's group at the European Molecular Biology Laboratory (EMBL). This microscope can realize high-speed z-axis scanning in deeper tissue by illuminating a specimen from the side with a light sheet (more information is given in Dr. Nonaka's section: Lab. for Spatiotemporal Regulations). Dr. Shigenori Nonaka conducted and supported about 10 projects as Collaborative Research Programs for Integrative Bioimaging. The IR-LEGO was developed by Drs. Shunsuke Yuba and Yasuhiro Kamei at the National Institute of Advanced Industrial Science and Technology (AIST). This microscope can induce a target gene of interest by heating a single target cell *in vivo* with a high efficiency irradiating infrared laser. Details are described in the next section. The IR-LEGO was also used for about 10 Collaborative Research projects, including applications for animals and plants.

Workshop and Symposium

In 2016 we held courses during both the 9th NIBB International Practical Course which focused on basic techniques for medaka and zebrafish research including imaging, and the 4th biological image processing training course. We also have been holding a "Bioimaging Forum" every year which discusses Bioimaging from various directions such as microscopy, new photo-technology, and computer science. This year we held the 10th NIBB Bioimaging Forum focused on adaptive optics for microscopy. In addition, we held three symposiums focused on new emerging model animals, next generation research using amphibians, and heat and temperature in biology.

Publication List on Cooperation

〔**Original papers (Selected)**〕

- Okada, K., Inohaya, K., Mise, T., Kudo, A., Takada, S., and Wada, H. (2016). Reiterative expression of pax1 directs pharyngeal pouch segmentation in medaka. Development *143*, 1800-1810.
- Petroutsos, D., Tokutsu, R., Maruyama, S., Flori, S., Greiner, A., Magneschi, L., Cusant, L., Kottke, T., Mittag, M., Hegemann, P., Finazzi, G., and Minagawa, J. (2016). A blue-light photoreceptor mediates the feedback regulation of photosynthesis. Nature *537*, 563-566.
- Shikata, T., Matsunaga, S., Kuwahara, Y., Iwahori, S., and Nishiyama, Y. (2016). Light spectrum regulates cell accumulation during daytime in the raphidophyte Chattonella antiqua causing noxious red tides. J. Photochem. Photobiol. B: Biology *160*, 128-133.
- Suthaparan, A., Solhaug, K. A., Stensvand, A., and Gislerod, H.R. (2016). Determination of UV action spectra affecting the infection process of *Oidium neolycopersici*, the cause of tomato powdery mildew. J. Photochem. Photobiol. B: Biology *156*, 41-49.
- Utagawa, U., Higashi, S., Kamei, Y., and Fukamachi, S. (2016). Characterization of assortative mating in medaka: Mate discrimination cues and factors that bias sexual preference. Horm. Behav. *84*, 9-17.
- Yokoyama, R., Yamamoto, H., Kondo, M., Takeda, S., Ifuku, K., Fukao, Y., Kamei, Y., Nishimura, M., and Shikanai, T. (2016). Grana-localized proteins, RIQ1 and RIQ2, affect the organization of light-harvesting complex II and grana. Plant Cell *28*, 2261–2275.
- Zeng, C.W., Kamei, Y., Wang, C.T., and Tsai, H.J. (2016). Subtypes of hypoxia-responsive cells differentiate into neurons in spinal cord of zebrafish embryos after hypoxic stress. Biol. Cell *108*, 1–21.

● ***Research activity by Y. Kamei***

Specially Appointed Associate Professor: *KAMEI, Yasuhiro*
NIBB Research Fellow: *HATTORI, Masayuki*
Postdoctoral Fellow: *ANDO, Yoriko*
Technical Assistant: *CHISADA, Eriko*
ATSUMI, Urumi

Our research group promotes two cutting-edge microscope projects; "observation" and "manipulation" using optical and biological technologies. The aim of our "observation project" is deep-seeing in living organisms using adaptive optics (AO) which were well-developed in the field of astronomy as a key technology of large telescopes such as the Subaru telescope in Hawaii. Although observation using telescopes on the earth may be disturbed by fluctuations in the atmosphere, AO technology can cancel this disturbance. On the other hand, living materials have particular refractive indexes, therefore, some organelles act as disturbances of the ideal optical path for microscope observation just like the atmosphere does for telescopes. AO technology can also compensate for this disturbance by sensing and correcting wave fronts using a wave front sensor and deformable mirror. Hence, we developed a custom-made wide-field microscope equipped with an AO system for observation of living organisms in collaboration with Dr. Tamada in NIBB and Dr. Hayano in the National Astronomical Observatory of Japan (NAOJ) and got high-resolution bright field and fluorescent images of living cells. Our results indicated that improvement of optical resolution was restricted to a small area which is called the "isoplanatic patch" (Figure 1).

Second, the aim of our "manipulation project" is to control gene expression *in vivo*. Gene function analysis must be evaluated at the cell level *in vivo*. To achieve spatiotemporal-controlled gene expression we employed one of the stress responses, the heat shock response. The heat shock promoter

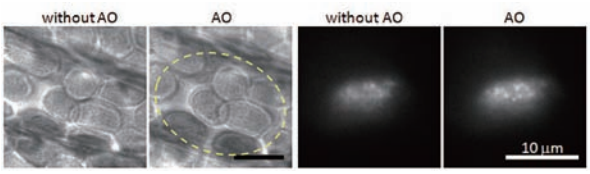


Figure 1. Effects of adaptive optics (AO) to wide-field microscope images (bright field and fluorescence of plant cells).

is the transcription regulation region of heat shock proteins and all organisms have this mechanism. Positioning the target gene downstream of the promoter, we can induce the target gene expression by local heating.

Infrared (IR) beams can heat water molecules, which are the main constituent of cells, hence, we can heat a single cell by irradiating IR to a target cell using a microscope. We have developed a microscope, IR laser evoked gene operator (IR-LEGO), specialized for this purpose (Figure 2). The IR-LEGO microscope can irradiate an IR laser to a single cell *in vivo* such as in *C. elegans*, *Drosophila*, medaka, zebrafish, *Xenopus* and *Arabidopsis*, to induce the heat shock response at a desired timing. In 2015, additionally, we confirmed the system was effective in the moss *Marchantia polymorpha* and in the newt *Pleurodeles waltl*.

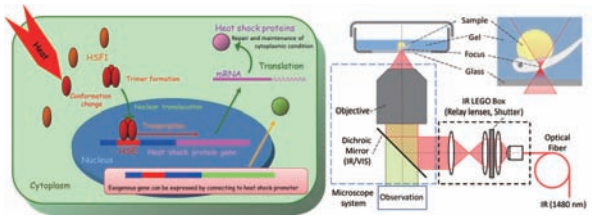


Figure 2. Schematic illustration of heat shock response of cells (left) and an infrared laser-evoked gene operator (IR-LEGO) microscope system.

Optimal heating induces the heat shock response and subsequent gene expression, while an excess results in cell death. Hence, we must precisely control laser heating. We evaluated time course and spatial heating profiles, and the results presented that temperature of the target area rose rapidly and kept a constant level dependant on IR laser power, additionally, the heated area was adequately as small as a typical cell size.

With this in mind, we tried to induce gene expression in various species. At first, we reported an IR-LEGO experiment in living *C. elegans*. Target gene expression in a target cell could be induced with only 1 s-IR irradiation. Whereas the optimal power range which can induce gene induction without cell damage was limited. Excess laser power resulted in cell death or cessation of cell division. We confirmed that an optimal irradiation, e.g. 11 mW for 1 s, induced physiological gene expression in the target cell and subsequent cell division or morphogenesis underwent normal development. Next, we tried the experiment in other animals, such as, medaka, zebrafish and *Xenopus*, and the higher plant, *Arabidopsis*, since all organisms have a heat shock response system. We succeeded in local gene induction in all the species as expected. Moreover, this system can be combined to the cre/loxP recombination technique for long-term gene

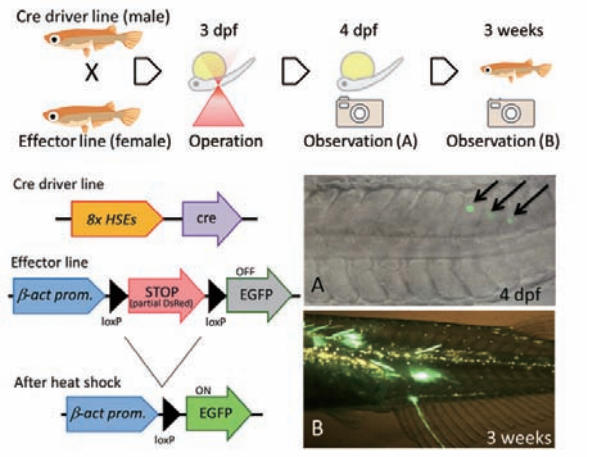


Figure 3. Long-term gene expression system with cre/loxP recombination system and its example of practical experiment in medaka embryo to adult.

expression (Figure 3).

As mentioned above, excess irradiation resulted in cell damage, so we utilized the system to ablate target cells with strong pulsed irradiation. In collaboration with National Taiwan University, we used the system for neuronal regeneration study in zebrafish and revealed that a kind of neuronal precursor cell played an important role within the neuron regeneration step the in zebrafish spinal cord (Zeng et al. Biol. Cell 2016). In addition, the IR-LEGO system can be utilized for thermo-biology because spatiotemporal micrometer order local heating is difficult without this system. Now we are trying to estimate thermal properties of cells and biomaterials *in vivo* using the IR-LEGO system.

Publication List:

〔Original papers〕

- Nishihama, R., Ishida, S., Urawa, H., Kamei, Y., and Kohchi, T. (2016). Conditional gene expression/deletion systems for Marchantia polymorpha using its own heat-shock promoter and the cre/loxP-mediated site-specific recombination. Plant Cell Physiol. *57*, 271-280.
- Suzuki, M., Takagi, C., Miura, S., Sakane, Y., Suzuki, M., Sakuma, T., Sakamoto, N., Endo, T., Kamei, Y., Sato, Y., Kimura, H., Yamamoto, T., Ueno, N., and Suzuki, K.T. (2016). In vivo tracking of histone H3 lysine 9 acetylation in *Xenopus laevis* during tail regeneration. Genes Cells *21*, 358-369.
- Utagawa, U., Higashi, S., Kamei, Y., and Fukamachi, S. (2016). Characterization of assortative mating in medaka: Mate discrimination cues and factors that bias sexual preference. Horm. Behav. *84*, 9-17.
- Yokoi, S., Ansai, S., Kinoshita, M., Naruse, K., Kamei, Y., Young, L.J., and Takeuchi, H. (2016). Mate-guarding behavior enhances male reproductive success via familiarization with mating partners in medaka fish. Front. Zool. *13*, 21.
- Yokoyama, R., Yamamoto, H., Kondo, M., Takeda, S., Ifuku, K., Fukao, Y., Kamei, Y., Nishimura, M. and Shikanai, T. (2016). Grana-localized proteins, RIQ1 and RIQ2, optimize the dynamics of light-harvesting complex II and grana stacking in Arabidopsis. Plant Cell *28*, 2261-2275.
- Zeng, C.W., Kamei, Y., Wang, C.T., and Tsai, H.J. (2016). Subtypes of hypoxia-responsive cells differentiate into neurons in spinal cord of zebrafish embryos after hypoxic stress. Biol. Cell, *108*, 357-377.

Data Integration and Analysis Facility

Assistant Professor: *UCHIYAMA, Ikuo*
Technical Staff: *MIWA, Tomoki*
NISHIDE, Hiroyo
NAKAMURA, Takanori
Technical Assistant: *OKA, Naomi*

The Data Integration and Analysis Facility supports research activities based on large-scale biological data analysis, such as genomic sequence analysis, expression data analysis, and imaging data analysis. For this purpose, the facility maintains high-performance computers with large-capacity storage systems. On the basis of this system, the facility supports development of data analysis pipelines, database construction and setting up websites to distribute the data worldwide as well as providing users' basic technical support. In addition to computational analysis, the Data Integration and Analysis Facility supports NIBB's information infrastructure, the maintenance of the network systems in the institute and computer/network consultation for institute members.

Representative Instruments

Our main computer system is the Biological Information Analysis System (BIAS) (Figure 1), which consists of a high-performance cluster system (SGI Rackable server C2112-4RP; 40 nodes/800 cores, 96GB memory/node), a shared memory parallel computer (HP ProLiant DL980 G7; 80 cores, 4TB memory), a high-throughput storage system (DDN SFA7700; 480TB), and a large capacity storage system (DELL PowerEdge R620; 720TB). All subsystems are connected via a high-speed InfiniBand network so that large amounts of data can be processed efficiently. Some personal computers and color printers are also available. On this system, we provide various biological databases and data retrieval/analysis programs, and support large-scale data analysis and database construction for institute members and collaborative researchers. Especially, we have supported the construction and maintenance of published databases of various model organisms including XDB (*Xenopus laevis*), PHYSCObase (*Physcomitrella patens*), DaphniaBASE (*Daphnia magna*), The Plant Organelles Database, and MBGD (microbial genomes).

The facility also provides network communication services. Most of the PCs in each laboratory, as well as all of the above-mentioned service machines, are connected by a local area network, which is linked to the high performance backbone network ORION connecting the three research institutes in Okazaki. Many local services, including sequence analysis services, file sharing services, and printer services, are provided through this network. We also maintain a public World Wide Web server that hosts the NIBB home page (http://www.nibb.ac.jp/en).

Figure 1. Biological Information Analysis System

Research activity by I. Uchiyama

Assistant professor I. Uchiyama is the principal investigator of the Laboratory of Genome Informatics, which currently focuses on microbial comparative genomics studies. For details, please refer to the laboratory page (p. 71).

NIBB BioResource Center

Head
FUJIMORI, Toshihiko

To understand the mechanisms of living organisms, studies focusing on each gene in the design of life (the genome) are required. The use of model animals and plants, such as mice, Medaka (*Oryzias latipes*), zebrafish, *Arabidopsis*, *Lotus japonicus*, and *Physcomitrella patens*, make it possible to produce genetically controlled organisms with markers placed using genetic and cell engineering technology. Such marking allows detailed studies of genes and cell functions. The model organisms mature in a short period of time; therefore, changes in cells, organs, and individuals can be totally and efficiently observed. The NIBB BioResource Center has equipment, facilities, and staff to maintain such organisms safely, efficiently, and appropriately.

Model Animal Research Facility

Specially Appointed Professor:	*NARUSE, Kiyoshi*
Associate Professor:	*WATANABE, Eiji*
	TANAKA, Minoru
Technical Staff:	*OHSAWA, Sonoko*
	HAYASHI, Kohji
	NOGUCHI, Yuji
Technical Assistant:	*TAKAGI, Yukari*
	SUZUKI, Kohta
	SUGINAGA, Tomomi
	FUJIMOTO, Daiji
	MATSUMURA, Kunihiro
	GODA, Misato
	NOMOTO, Yoshihiro

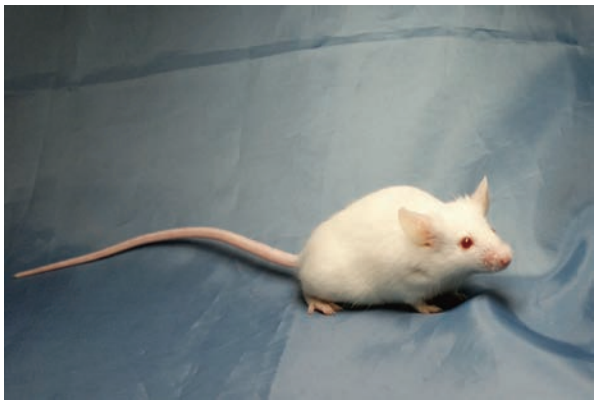


Figure 1. Mouse (MCH(ICR))

The worldwide genome project has almost been completed and research on basic biology has arrived at a post-genome era in which researchers are focusing on investigating the functions of individual genes. To promote the functional analysis of a gene of interest it is essential to utilize genetically altered model organisms generated using genetic engineering technology, including gene deletion, gene replacement and point mutation.

The NIBB Center for Transgenic Animals and Plants was established in April 1998 to support research using transgenic and gene targeting techniques at NIBB. The NIBB Center for Transgenic Animals and Plants was integrated into the NIBB BioResource center in April 2010, and was renamed "The Model Animal Research Facility".

Technical and supporting staff develop and promote research-supporting activities. A state-of-the-art facility for transgenic animals opened at the end of 2003 in the Yamate area.

The activities of the model animal research facility are as follows:

1. The provision of information, materials, techniques, and animal housing space to researchers.
2. The use of various kinds of instruments to analyze mutant, transgenic, and gene-targeted animals.
3. The development of novel techniques related to transgenic and gene targeting technology.
4. Cryopreservation and storage of transgenic strains.

I. Research support activities (mouse)

In 2001, the NIBB mouse facility (built under specific pathogen free (SPF) conditions) opened in the Myodaiji area and the production, breeding, analysis, cryopreservation and storage of genetically manipulated mouse strains has been conducted there since. The new center facility building in the Yamate area strengthened research activities using genetically altered organisms. The building has five floors and a total floor space of 2,500 m^2 in which we can generate, breed, store and analyze transgenic, gene targeting, and mutant mice under SPF conditions. The mouse housing area was constructed based on a barrier system. This building is also equipped with breeding areas for transgenic small fish and birds.

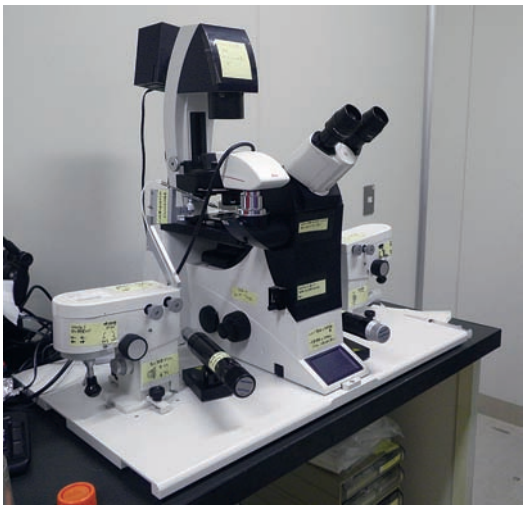


Figure 2. Equipment for manipulating mice eggs.

In 2016 (from January 1 to December 31), 2,907 fertilized eggs (*in vitro* fertilization; 2,546 eggs of 14 lines in which 1,747 eggs of 9 lines were frozen for long-term storage, frozen eggs: 361 of 7 lines) and 4,175 mice were brought

into the facility in the Yamate area, and 59,019 mice (including pups bred in the facility) were taken out.

A number of strains of genetically altered mice from outside the facility were brought into the mouse housing area by microbiological cleaning using *in vitro* fertilization-embryo transfer techniques, and stored using cryopreservation.

A new mouse facility in the Myodaiji area opened at the beginning of 2005. The facility provides research-supporting activities to researchers in the Myodaiji area. In March 2009, we expanded the facility which includes areas for breeding, behavioral tests, and transgenic studies using various kinds of recombinant viruses. In 2016 (from January 1 to December 31), 25 mice were brought into the facility in the Myodaiji area, and 1,961 mice (including pups bred in the facility) were taken out.

Figure 3. Large sized autoclave in the Myodaiji area.

II. Research support activities (small fish and birds)

The first floor of the center facility building in the Yamate area provides space and facilities to maintain small fish and chick embryos. In the laboratory room for chick embryos, a large incubation chamber is equipped and set at 42 degrees (suitable for chick embryogenesis). The researchers can manipulate chick embryos under optimal conditions, removing biohazard risks. For researchers who employ fish as an experimental model, 480 tanks (1 liter) and 450 tanks (3 liters) are available for medaka and zebrafish, respectively. Water circulates and can be maintained to suit the conditions desired for fish breeding in the aquarium systems. Currently, over three mutant lines and over fifteen transgenic lines of medaka and zebrafish are maintained in our facility. A medaka line that allows gene induction by heat treatment, in combination with a cre/loxP system, has been developed in this facility. All the rooms are qualified to meet the criteria for transgenic animals, allowing researchers to generate and maintain these important biological tools.

In 2016 (from January 1 to December 31), 600 medaka (300 adults and 300 fertilized eggs) were brought to the facility and 23,691 medaka and zebrafish (2,796 adults, 90 embryos and 20,805 fertilized eggs, including animals bred in the facility) were taken out. In the laboratory for chick embryos there were no fertilized eggs or chicken embryos brought in or taken out this year. These animals were used for research activities in neurobiology and developmental biology.

In 2007 NIBB was approved as a core facility of the National BioResource Project (NBRP) for Medaka by the Japanese Government. We have supported the activities of NBRP Medaka by providing standard strains, mutants, transgenic lines and organizing international practical courses for medaka. In 2014 we began providing the CRISPR/Cas9 genome editing platform as well as the TILLING library screening system to promote the reverse genetic approach. In 2015 we shipped 220 independent medaka strains, 155 cDNA/BAC/Fosmid clones, and 195 samples of hatching enzyme to the scientific community worldwide.

Figure 4. Quarantine room for medaka and zebrafish.

III. Research activities

The associate professors of this center - E. Watanabe, T. Naruse, and M. Tanaka - are the principal investigators of the Laboratory of Neurophysiology, the Laboratory of Bioresources, and the Laboratory of Molecular Genetics for Reproduction, respectively. The Laboratory of Neurophysiology is studying mechanisms of the visual system using a psychophysical approach. The Laboratory of Bioresources has conducted a genetic and genomic analysis of quantitative traits and Mendelian phenotype variations, as well as evolution of sex determination systems in medaka related species. The Laboratory of Molecular Genetics for Reproduction is studying the molecular mechanisms of reproductive organ development and sex differentiation using mutagenized or transgenic medaka. For details, please refer to the pages of each laboratory (p. 42, 51, and 33).

Model Plant Research Facility

● *Plant Culture Laboratory*

Assistant Professor:	*HOSHINO, Atsushi*
	TSUGANE, Kazuo
Technical Staff:	*MOROOKA, Naoki*
Technical Assistant:	*SUZUKI, Keiko*
	KOTANI, Keiko

The Plant Culture Laboratory manages facilities for the cultivation of plants in general and also for the rearing of several animal species that do not fit in other facilities.

The Plant Culture Laboratory equips and manages 62 culture boxes or growth chambers, and 12 rooms with the P1P physical containment level for established and emerging model plants, for example the thale cress *Arabidopsis thaliana*, the rice *Oryza sativa*, the moss *Physcomitrella patens*, the liverwort Marchantia polymorpha, the green alga *Chlamydomonas reinhardtii* and several other flowering plants including several carnivorous plants. Most culture space is fully used the whole year by more than 80 researchers from both outside and inside groups.

As well as regular culture conditions, extreme environmental conditions for light and temperature are available for various types of experiments. Three chambers (3.4 m^2 each) that can control CO_2 and humidity in addition to temperature and light (max 70,000 lux) conditions are available. A tissue culture rack with dimming LEDs and pulse-width modulation controllers are used for algae culture under precise light control. Autotrophic and heterotrophic culture devices are also available for researchers using cyanobacteria, algae, and cultured flowering plant cells. Aseptic experiments can be performed in an aseptic room with clean benches and a safety cabinet. Several analytical instruments including a flow cytometry system and a DUAL-PAM, for DNA content and chlorophyll fluorescent measuring, respectively, are also available.

Next to the institute building of the Myodaiji area, a 386-m^2 experimental farm is maintained for Japanese morning glory and related *Ipomoea* species, several carnivorous plants and other flowering plants necessary to be cultivated outside. Three greenhouses (44, 44, and 45 m^2) with heating are used for the sensitive carnivorous plants. Four greenhouses (4, 6, 9, and 9 m^2) with air-conditioning are provided for the cultivation of rice *Oryza* sp., *Lotus japonica* and related legume species, as well as mutant lines of the Japanese morning glory. Two greenhouses (9 and 18 m^2) with air-conditioning meet the P1P physical containment level and are available for experiments using transgenic plants. The Plant Culture Laboratory also maintains a 46 m^2 building with storage and workspace. Part of the building is used for rearing of the orchid mantis and the Japanese rhinoceros beetle.

Due to the building renovation work, 4 phytotrons and three light environmental simulators were removed, and some of the facilities are temporally closed. The closed facilities will be renewed and opened in May 2017.

● *Morning Glory BioResource Laboratory*

Assistant Professor:	*HOSHINO, Atsushi*

The Japanese morning glory (*Ipomoea nil*) is a traditional floricultural plant in Japan, and is studied worldwide, especially in plant physiology and genetics. NIBB collects, develops and distributes DNA clones, mutant lines for flower pigmentation, and transgenic lines as a sub-center of the National BioResource Project (NBRP) Morning glory, and collaborates with the core organization center, Kyushu University. We collected several mutant lines, and provided 47 DNA clones to both local and international biologists this year. A database for the whole genome sequence of *I. nil* was built and opened to the public.

Research activities of the Assistant Professor A. Hoshino are shown on the laboratory page (p. 58).

Cell Biology Research Facility

Associate Professor:	*WATANABE, Eiji*
Assistant Professor:	*HAMADA, Yoshio*
Technical Assistant:	*SUGINAGA, Tomomi*

The Cell Biology Research Facility provides various equipment for tissue and cell culture. This laboratory is equipped with safety rooms which satisfy the P2 physical containment level, and is routinely used for DNA recombination experiments.

Figure 5. Equipment for tissue and cell culture.

NIBB Center of the Interuniversity Bio-Backup Project (IBBP Center)

Head
KAWAGUCHI, Masayoshi

Specially Appointed Professor
NARUSE, Kiyoshi

Specially Appointed Assistant Professor:
KIMURA, Tetsuaki
TAKETSURU, Hiroaki

Technical Assistant:
AKIMOTO-KATO, Ai
MATSUBAYASHI, Naomi
MIZOKAMI, Yuko
TSUZUKI, Chizuru

In order to realize a life sciences community resilient to natural disasters and calamities, the National Institutes for Natural Sciences (NINS) and Hokkaido University, Tohoku University, University of Tokyo, Nagoya University, Kyoto University, Osaka University, and Kyushu University concluded an agreement on June 1st 2012 to launch a system to 'back up' the biological resources essential to the work being done at universities and research institutions nationwide, called the 'Interuniversity Bio-Backup Project (IBBP)'.

The IBBP Center was established as a centralized backup and storage facility at NIBB, while IBBP member universities set up satellite hubs and work closely with the IBBP center to put in place reciprocal systems for backing up important biological resources that have been developed by researchers residing in the area each university satellite hub is responsible for.

Figure 1. IBBP Center.

Figure 2. Cryogenic storage system. Liquid nitrogen tanks are monitored 24 hours a day and are refilled automatically.

The IBBP Center includes earthquake proof structures capable of withstanding even very large scale quakes (equipped with emergency backup power generators), cryopreservation facilities equipped with automatic liquid nitrogen feeding systems, deep freezers, and refrigerated storage (mainly for seed stocks), as well as all manner of automated laboratory equipment, cell culture tools, and the latest equipment necessary to back up the genetic resources in a collaborative manner. The specific methods of preservation are freezing of the sperm and eggs of animals, cultured plant and animal cells, proteins and gene libraries. Plant seeds are frozen or refrigerated.

University satellite hubs receive preservation requests of biological resources from researchers and report to the Managing Project Committee of IBBP (constituted of faculty of NIBB and satellite institutes), where the relevance of the request is reviewed. When the request is sustained, biological resources to be preserved will be sent to the IBBP Center by the requesting researcher, where they will be frozen (or refrigerated) and their particular information will be registered into a database. In the event of a disaster leading to the loss of a researcher's own biological resources, preserved samples will be promptly supplied back to the researcher so they can quickly resume their work.

Through the development of this backup system biological resources that had only been stored at individual research institutes can now be preserved at the state of the art facilities of the IBBP Center, and Japan's research infrastructure has been significantly strengthened.

I. Current status of back up for the biological resources

In 2016, IBBP Center stored 4,478 384-well and 69 96-well plates consisting of 1,726,176 clones as cDNA/BAC clones, 11,792 tubes for animal cells, plant and animal samples, proteins, genes and microorganisms, 541 133mm-straw tubes for sperm and 654 seed samples. In total 1,727,371 samples are stored.

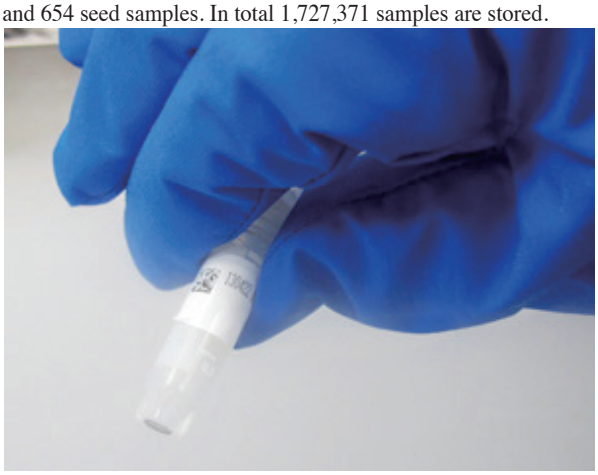


Figure 3. Cryo tube with 2D barcode. Each sample is printed with a unique barcode and is managed using a database.

II. Collaborative Research Project for the development of new long-term storage technologies and cryo-biological study

As the IBBP Center can only accept biological resources which can be cryopreserved in liquid nitrogen, researchers cannot backup those biological resources for which cryo-preservation methods are not well established, to increase the usability of IBBP, we started a collaborative research project for the development of new long-term storage technologies and cryo-biological study in 2013. This collaborative research includes two subjects 1) Establishment of new storage technologies for biological resources for which long-term storage is unavailable. 2) Basic cryobiological research enabling us to improve low temperature storage for biological resources. In 2016, we also worked to establish a research center for cryo-biological study thorough this Collaborative Research Project. Accordingly, we organized the Cryopreservation Conference 2016 on November 10-11, 2016 at the Okazaki Conference Center, Okazaki, Japan. We had 128 participants from several fields covering physics, chemistry, biology, and technology.

Research activity by H. Taketsuru

Specially Appointed Assistant Professor:
TAKETSURU, Hiroaki

In vitro maturation of immature rat oocytes

Rat oocytes can be produced artificially by superovulation. Because some strains show low sensitivity to superovulation treatment, *in vitro* maturation is an alternative method to produce numerous matured oocytes. Furthermore, establishment of an *in vitro* maturation system with simple culture conditions is cost effective and leads to easy handling of oocytes. This study examined developmental ability of rat germinal vesicle (GV) oocytes maturing *in vitro* under simple culture conditions. Significantly different numbers of ovulated oocytes reached the second metaphase of meiosis (MII) among Jcl:Wistar (17.0), F344/Stm (31.0), and BN/SsNSlc (2.2) rats in whom superovulation was induced by pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (Figure 1). However, similar numbers of GV oocytes were obtained from ovaries of PMSG-injected

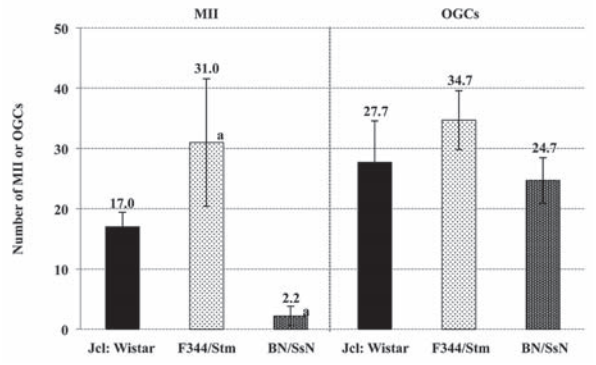


Figure 1. Number of MII oocytes or OGCs collected from ovaries of females. [a]Significantly different at $P < 0.05$.

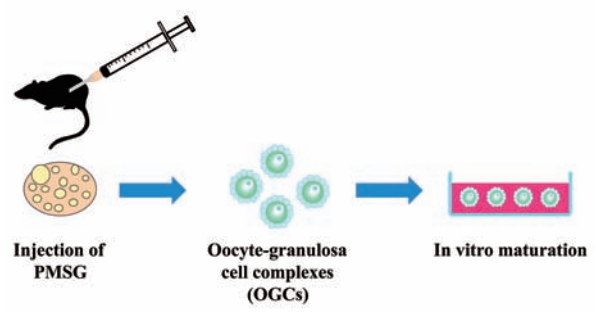


Figure 2. In vitro maturation of immature rat oocytes.

Wistar (27.7), F344 (34.7), and BN (24.7) rats (Figure 1). These GV oocytes were cultured *in vitro* in HTF, αMEM, and a 1:1 HTF+αMEM or TYH+αMEM mixture (Figure 2). High proportions of Wistar and F344 oocytes that matured to MII in αMEM were parthenogenetically activated by strontium chloride treatment (78% and 74%, respectively). Additionally, 10% of matured oocytes of both strains developed into offspring after intracytoplasmic sperm injection and embryo transfer to foster mothers (Figure 3). Although BN oocytes cultured in αMEM could be parthenogenetically activated and developed into offspring, the success rate was lower than that for Wistar and F344 oocytes. This study demonstrated that numerous GV oocytes were produced in rat ovaries by PMSG injection. This simple *in vitro* maturation system of immature oocytes could be further developed to maintain valuable rat strains experiencing reproductive difficulties.

Figure 3. Offspring were obtained from IVM oocytes.

Publication List:

〔**Original paper**〕

● Taketsuru, H., and Kaneko., T. (2016). In vitro maturation of immature rat oocytes under simple culture conditions and subsequent developmental ability. J. Reprod. Dev. *62*, 521-526.

Research activity by T. Kimura

Specially Appointed Assistant Professor:
KIMURA, Tetsuaki

Positional cloning of iridophores mutant

See-through medaka lines are suitable for the observation of internal organs throughout life. They were bred by crossings of multiple color mutants. However, some of the causal genes for these mutants have not been identified. The medaka has four pigment cell types: black melanophores, yellow xanthophores, white leucophores, and silvery iridophores. To date, causal genes of melanophore, xanthophore, and leucophore mutants have been elucidated, but the causal gene for the iridophore mutant remains unknown. Iridophore mutant *guanineless* (*gu*) exhibits a strong reduction in visible iridophores throughout its larval and adult stages. The *gu* locus was previously mapped on chromosome 5, but was located near the telomeric region, making it difficult to integrate into the chromosome. To circumvent this, I perfomed synteny analysis using the zebrafish genome and found a strong candidate gene, *pnp4a*. Gene targeting and a complementation test showed that the candidate gene is a causal gene of gu. This result will allow the establishment of inbred medaka strains or other useful strains with see-through phenotypes, without disrupting the majority of the genetic background of each strain.

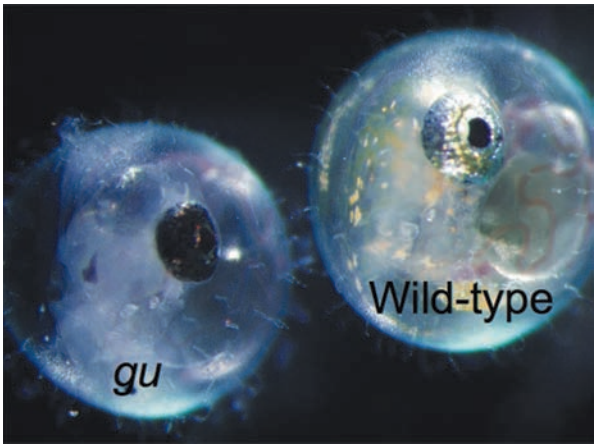


Figure 1. *gu* and wild-type.

CENTER FOR RADIOISOTOPE FACILITIES

Head
HASEBE, Mitsuyasu

Associate Professor
KODAMA, Ryuji

Technical Staff: *MATSUDA, Yoshimi (Radiation Protection Supervisor)*
SAWADA, Kaoru (Radiation Protection Supervisor)
IINUMA, Hideko

Technical Assistant: *HAYASHI, Tomoko*

The technical and supporting staff of the Center for Radioisotope Facilities (CRF) maintain controlled areas in compliance with the law. The CRF is responsible for monitoring the purchase of radioisotopes from the Japan Radioisotope Association (JRIA) and the transfer of radioisotope wastes to JRIA.

Ms. Matsuda, Ms. Iinuma and Ms. Hayashi maintained the Myodaiji area. Ms. Sawada worked in the Yamate area. Dr. Kodama worked in both areas.

The following are the CRF's notable activities in 2016.

1. The facility's emergency manual was updated. Main points of modification are the following.

1) When the fire alarm goes off.

2) When the warning declaration about an earthquake disaster is issued.

The emergency manual file is placed in the contamination check room.

2. The cameras of the video recording system at the Yamate area were superannuated, and were exchanged in February 2016. (Figure 1A)

3. The pumps of the radioisotope waste water treatment facility at the Myodaiji area could not start draining when the water level was low. By adding an electric valve and exchanging flap valves in February 2016, the pumps operate normally. (Figure 1B)

4. As the number of users decreases, the ventilating installations, the air conditioners, and the low temperature rooms are deactivated when there are no users. The ice makers were deactivated after June 2016.

The number of registrants and the number of users from January 2016 to December 2016 are shown in Table 1.

Figure 1. The CRF's notable activities in 2016.
A: exchanging of the video camera at the Yamate area
B: adding the electric valve at the Myodaiji area

Users and visitors counted by the access control system of the controlled areas numbered 1,531 during this period. The numbers for each area are shown in Table 2. The annual changes of registrants and the number of totals per fiscal year are shown in Figure 2. The balance of radioisotopes received and used at the CRF is shown in Table 3. The training courses on radioisotope handling were given as in Table 4.

	Myodaiji Area	Yamate Area
Registrants	55	54
Users	27	23

Table 1. Numbers of registrants and users at Myodaiji area and Yamate area in 2016.

	Myodaiji Area	Yamate Area	Total
Users	851	430	1,281
Visitors	132	118	250
Total	983	548	1,531

Table 2. Users and visitors who entered each controlled area in 2016.

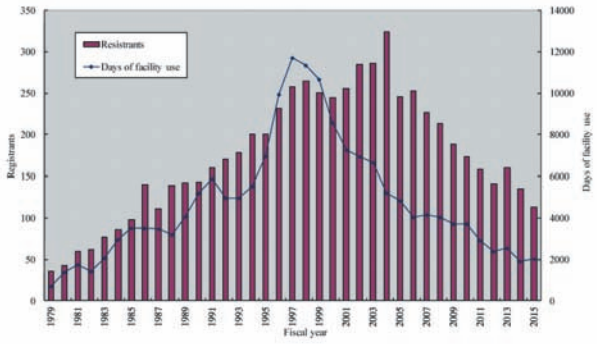


Figure 2. Annual changes of registrants and days of facility use per fiscal year.

	Myodaiji Area	Yamate Area	Total
^{125}I Received	0	3,866	3,866
^{125}I Used	0	1,072	1,072
^{35}S Received	0	0	0
^{35}S Used	0	0	0
^{32}P Received	249,750	0	249,750
^{32}P Used	193,051	0	193,051
^{14}C Received	37,000	0	37,000
^{14}C Used	6,000	0	6,000
^{3}H Received	0	925,000	925,000
^{3}H Used	0	1,110	1,110

Table 3. Balance of radioisotopes received and used (kBq) at each controlled area in 2016.

training course	place	numbers of participant
Introductory course for beginners	Myodaiji	1
Introductory course for beginners	Yamate	1
Introductory course for experts	Myodaiji	3
Introductory course for experts	Yamate	2
Users training course*	Myodaiji	48
Users training course	Yamate	43

*including English course

Table 4. Training courses for radiation workers in 2016.

RESEARCH ENHANCEMENT STRATEGY OFFICE

Director
UENO, Naoto

Vice-Director (Specially Appointed Professor (URA))
NISHIMURA, Mikio

In order to fulfill two goals, to encourage cutting-edge academic research in the field of natural sciences through international joint research, and to contribute to the enhancement of research capabilities of universities etc. in Japan using the world's most advanced research environment for joint utilization and joint research, NINS started in 2013 a research enhancement project with the following four approaches: 1) Support for the promotion of international advanced research, 2) Support for the promotion of joint utilization and joint research in Japan, 3) Dissemination of information and enhancement of public relations in Japan and abroad, 4) Support for researchers, especially young, female or foreign researchers.

The Research Enhancement Strategy Office is aimed at supporting researchers so that NIBB improves its ability as a collaborative research institution, and was restructured in 2013 from the former Strategic Planning Department, the Office of Public Relations, and the Office of International Cooperation which existed from 2005. The Office's activities are mainly carried out by URAs (University Research Administrators) according to the advice of the group adviser chosen from NIBB's professors and in close collaboration with the NINS's Research Enhancement Promotion Headquarters.

Evaluation and Information Group

Associate Professor:	*KODAMA, Ryuji*
Group Adviser:	*YOSHIDA, Shosei*

This group serves as a central office for assisting the Director General in preparing for NIBB's evaluation procedure and in planning long-range strategies for the institute.

The main activities of the group

1) Management of external evaluation processes

NIBB asks non-NIBB members of the Advisory Committee for Programming and Management to answer a questionnaire on research achievements and collaborative research activities of the institute. Several of the members are further invited to participate in a meeting for the evaluation of these activities and for proposing future directions for the institute. This group manages these processes.

2) Editing of the Annual Report (in collaboration with the Public Relations Group)

This group edits the annual report (this print) which summarizes the annual activities of laboratories and facilities and is used in the evaluation procedures of the institute.

3) Assistance in budget requests and long-range planning of the institute

This group also assists the Director General in preparing long-range plans for building the most advanced research facilities, and in budget requests to the government to realize and implement these plans.

4) Assistance in making the plans and reports of the institute

In addition we assist in drafting NIBB's Medium-term Goals and Plans (for a six-year-period), and in instituting Annual Plans to implement them. The department also assists in preparing Business and Performance Reports for the external evaluation on whether we are meeting the goals set both annually and for the medium-term.

Public Relations Group

Specially Appointed Assistant Professor (URA):	*KURATA, Tomoko*
Technical Assistant:	*OTA, Kyoko*
	KAWAGUCHI, Colin
	BAN, Misato
Group Adviser:	*FUJIMORI, Toshihiko*

This group, in order to publicize the activities of NIBB to the widest audience, actively facilitates communication between NIBB and the public, school teachers, and the international community of scientific researchers.

The main activities of the group in 2016

1) Press releases

The group sends news on scientific achievements to newspaper and magazine reporters via leaflets, e-mails and web pages. We also arrange press conferences for some releases.

2) Updating and maintenance of the NIBB web page

3) Editing of publications, production of posters and leaflets

Publication of "NIBB News" (Intra-institutional newsletter, in Japanese). Publication of leaflets introducing the individual researchers of NIBB (in Japanese). Design and distribution of posters of NIBB events.

4) Producing Videos

Creation of videos introducing NIBB and interviews with researchers that are published on the web.

5) Organization of scientific outreach programs

Organizing the Summer Program for university students, and coordinating special classes for middle school students.

INTERNATIONAL COOPERATION GROUP

Specially Appointed Assistant Professor (URA): *TATEMATSU, Kiyoshi*
Technical Assistant: *TAKAHASHI, Ritsue*
SANJO, Kazuko
NISHIMURA, Akiko
Group Advisor: *UENO, Naoto*

NIBB has a mission to continually explore the leading-edge of biology and form research communities that link Japan to the world. For this purpose, NIBB holds scientific meetings including "NIBB Conferences" and "Okazaki Biology Conferences (OBC)", and educational programs such as "NIBB International Practical Courses". Further, NIBB is tightly interacting with the European Molecular Biology Laboratory (EMBL, European member states), the Temasek Life Sciences Laboratory (TLL, Singapore) and Princeton University, USA on the basis of cooperative agreements, through exchanging people and techniques and jointly holding scientific meetings. NIBB is also conducting the "NIBB International Collaborative Research Initiative" to promote high-level international collaborations between faculty members of NIBB and researchers around the world. NIBB invites leading-edge researchers from abroad as "Guest Professors" to promote academic exchange with NIBB members and to start new international collaborations.

This group supports and coordinates NIBB's activities related to international research collaborations, through organizing the various above-mentioned international scientific meetings and technical courses, coordination of dispatching NIBB's researchers to international conferences, and support of researchers visiting from the institutes mentioned above. This group also supports NIBB internship students visiting from foreign countries, and the dispatching of graduate students of SOKENDAI (the Graduate University for Advanced Studies) to international conferences, which are aimed at nurturing the next generation of researchers in biology. This group, cooperating with the Okazaki Administration Office and International Affairs Division, City of Okazaki, also supports researchers and students who visit NIBB.

The main activities of the group in 2016

1) Coordination of the International Conferences and the International Practical Course

This group coordinated the following International Conference hosted by NIBB:

The 64th NIBB Conference "Evolution of Seasonal Timers" Okazaki, Japan, April 22 - 24, 2016 (p. 93)

The 9th NIBB International Practical Course and the 4th NIBB-TLL Joint International Practical Course "Genetics and Imaging of Medaka and Zebrafish" Okazai, Japan, Augusut 18 – 31, 2016 (p. 94)

2) Support of visiting researchers to NIBB

This group supported visits of foreign researchers related to the following events:

One Guest Professor coming from Academia Sinica, Taiwan (March) with the financial support of NINS and two researchers coming from EMBL, Germany (July) and Princeton University, USA (September)

3) Support of education related programs

This group supported hosting students participating in the following student-related activities of NIBB:

NIBB Internship Program 2016 (p. 98)

Collaborative Research Group

Specially Appointed Associate Professor (URA): *SHIGENOBU, Shuji*
KAMEI, Yasuhiro
Technical Assistant: *ICHIKAWA, Mariko*
ICHIKAWA, Chiaki
Group Adviser: *YOSHIDA, Shosei*

Specially appointed associate professors of this group belong to the NIBB core research facilities and are responsible for managing collaborative research projects and practical courses taking advantage of their expertise in their field, through which this group explores further promotion of information exchange and collaboration among scientific communities, and also supports the development of new equipment and methods.

In 2016, this group hosted a total of 114 collaboration projects. Through these collaborations, 28 research papers were published. A noteworthy achievement was the award-winning publication by Prof. Wakabayashi (Tokyo Institute of Technology) and his colleagues on the phototactic response of the green alga *Chalmydomonas reinharditii*. Through the close collaboration, the NIBB team analyzed the genome of an alga mutant by using next-generation sequencing technology resulting in the identification of the gene responsible for the phenotype.

- Ide, T., Mochiji, S., Ueki, N., Yamaguchi, K., Shigenobu, S., Hirono, M., and Wakabayashi, K. (2016). Identification of the *agg1* mutation responsible for negative phototaxis in a "wild-type" strain of *Chlamydomonas reinhardtii*. Biochem. Biophys. Rep. *7*, 379-385.
- Ueki, N., Ide, T., Mochiji, S., Kobayashi, Y., Tokutsu, R., Ohnishi, N., Yamaguchi, K., Shigenobu, S., Tanaka, K., Minagawa, J., Hisabori, T., Hirono, M., and Wakabayashi, K. (2016). Eyespot-dependent determination of the phototactic sign in *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA *113*, 5299-5304.

Gender Equality Promotion Group

Associate Professor: *TSUBOUCHI, Tomomi*
Group Adviser: *TAKADA, Shinji*

This group supports the improvement of the research environment for female researchers in both research and daily life. This includes our research support system for childbirth and childcare, etc., aiming at promoting the employment of female researchers.

In 2016, this group contributed to the management of the research support system covering childbirth and childcare and to establishing a network between female researchers.

TECHNICAL DIVISION

Head
KAJIURA-KOBAYASHI, Hiroko

Common Facility Group

Chief: *MIWA, Tomoki*

● ***NIBB Core Research Facilities***

Unit Chief:	*KONDO, Maki*
Subunit Chief:	*MAKINO, Yumiko*
	YAMAGUCHI, Katsushi
Technical Staff:	*NISHIDE, Hiroyo*
	NAKAMURA, Takanori
	TANIGUCHI-SAIDA, Misako
	UCHIKAWA, Tamaki
	BINO, Takahiro
Technical Assistant:	*ICHIKAWA, Chiaki*
	NISHIMURA, Noriko
	ICHIKAWA, Mariko
	ISHIKAWA, Azusa
	OKA, Naomi
	SHIBATA, Emiko

● ***NIBB Bioresource Center***

Unit Chief:	*OHSAWA, Sonoko*
Subunit Chief:	*HAYASHI, Kohji*
	MOROOKA, Naoki
Technical Staff:	*NOGUCHI, Yuji*
Technical Assistant:	*TAKAGI, Yukari*
	SUZUKI, Keiko
	SUZUKI, Kohta
	SUGINAGA, Tomomi
	KOTANI, Keiko

● ***Disposal of Waste Matter Facility***

Unit Chief: *MATSUDA, Yoshimi*

● ***Center for Radioisotope Facilities***

Unit Chief:	*MATSUDA, Yoshimi*
Subunit Chief:	*SAWADA, Kaoru*
Technical Staff:	*IINUMA, Hideko*
Technical Assistant:	*ITO, Takayo*

Research Support Group

Chief: *MORI, Tomoko*

● ***Developmental Biology***

Technical Staff:	*TAKAGI, Chiyo*
	UTSUMI, Hideko
	OKA, Sanae
	MIZUGUCHI, Hiroko

● ***Neurobiology***

Subunit Chief: *TAKEUCHI, Yasushi*

● ***Evolutionary Biology and Biodiversity***

Unit Chief:	*FUKADA-TANAKA, Sachiko*
Unit Chief:	*MIZUTANI, Takeshi*
Subunit Chief:	*KABEYA, Yukiko*

● ***Environmental Biology***

Technical Staff: *NODA, Chiyo*

Reception

Secretary:	*TSUZUKI, Shihoko*
	KATAOKA, Yukari
	UNO, Satoko
	MIYATA, Haruko

The Technical Division is a support organization for researchers and research organizations within NIBB. The Technical Division develops and promotes the institute's research activities and maintains the research functions of the institute.

The Technical Division is organized into two groups: the Common Facility Group, which supports and maintains the institute's common research facilities, and the Research Support Group, which assists research activities as described in the reports of individual research divisions.

Technical staff members continually participate, through the Division, in self-improvement and educational activities to increase their capabilities and expertise in technical areas. Generally, technical staff members are attached to specific divisions so that they may contribute their special biological and biophysical knowledge and techniques to various research activities.

The Technical Division hosts an annual meeting for technical engineers who work in various fields of biology at universities and research institutes throughout Japan. At this meeting, the participants present their own activities and discuss technical problems. The proceedings are published after each meeting.

The 64th NIBB Conference “Evolution of Seasonal Timers”

Organizers: Andrew Loudon (Univ. Manchester, UK), David Burt (Roslin Inst., UK), David Hazlerigg (UiT –Arctic Univ. Norway, Norway), Takashi Yoshimura (NIBB, Japan)

April 22 (Fri) - 24 (Sun), 2016

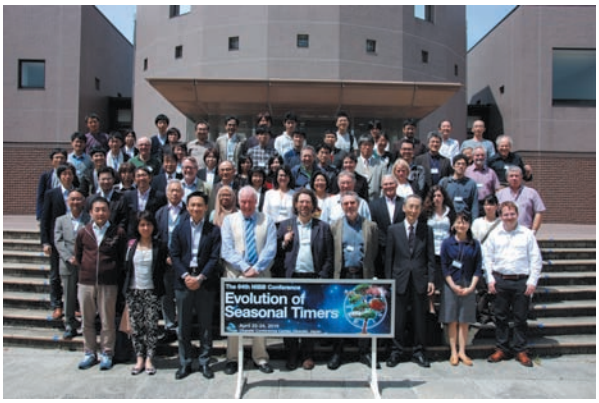
Until now how animals recognize and adapt to seasonal changes in the environment has remained a mystery that has long fascinated human beings. In addition, the impact of sudden climate change caused by global warming etc. on the ecosystem is also a concern, and societal interest in this mystery is great.

With recent research understanding of the mechanisms by which plants and animals perceive seasons has progressed, and both the universality and diversity of the seasonal sensing mechanisms of living organisms has come to light. With this background, the 64th NIBB Conference entitled “Evolution of seasonal timers” was held with the aim of promoting in-depth understanding of the design principles and evolution of the seasonal sensing mechanisms of living things.

Although the keyword of the conference was “season”, topics ranged from “mathematical analysis of the flowering of wild plants in the field”, “mechanisms of migratory behavior and spawning activity of eels”, “hibernation of squirrels”, and “chromatin remodeling and chemical genetics”. Leading researchers in their respective fields came together from across the world. The approximately 100 participants made for an ideal size for the meeting, and facilitated deep discussions in face-to-face settings for every participant throughout the three day period. Members who would normally never have a chance to meet face to face at a usual academy gathered together for an opportunity to deeply consider seasonal sensation from an interdisciplinary point of view, and we were able to obtain a lot of inspiration toward future research. We received kind acknowledgements such as “the most stimulating symposium I ever had” and “an unforgettable symposium”

I’d like to express my gratitude to the members of the Office of International Cooperation and the Division of Seasonal Biology for the efforts they made to the conference, and I very much appreciate the support from the National Institute for Basic Biology, the Daiko foundation, and the Human Frontier Science Program.

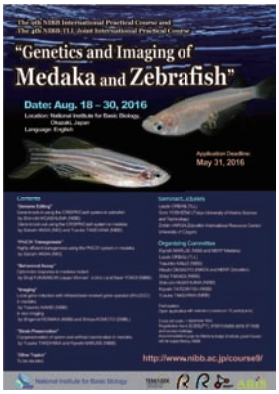
Takashi Yoshimura (On behalf of the organizers)



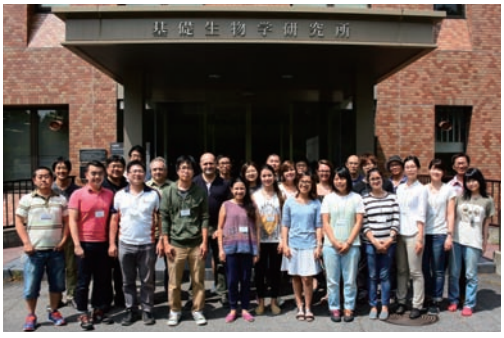
Speakers

Barnes, Brian (UAF), Burt, David (The Roslin Institute/Univ. of Edinburgh), Foulkes, Nicholas (KIT), Hazlerigg, David (UiT The Arctic Univ. of Norway), Loudon, Andrew (The Univ. of Mancheste), McClung, C. Robertson (Dartmouth College), Menaker, Michael (Univ. of Virginia), Simonneaux, Valerie (INCI), Tessmar-Raible, Kristin (Univ. of Vienna)
Izawa, Takeshi (The Univ. of Tokyo), Kitano, Jun (NIG), Kudoh, Hiroshi (Kyoto Univ.), Myung, Jihwan (RIKEN), Nakamichi, Norihito (Nagoya Univ.), Niwa, Ryusuke (Univ. of Tsukuba), Numata, Hideharu (Kyoto Univ.), Satake, Akiko (Kyushu Univ.), Takumi, Toru (RIKEN), Tsukamoto, Katsumi (Nihon Univ.), Yoshimura, Takashi (NIBB)

The 9th NIBB International Practical Course, The 4th NIBB-TLL Joint International Practical Course "Genetics and Imaging of Medaka and Zebrafish"



- Period: August 18 (Thu) - 31(Wed), 2016
- Participants: 17 (2 from Argentina, 2 Korea, 2 Singapore, 1 Germany, 1 India, 1 Taiwan, 1 UK, and 7 Japan)
- Venue: National Institute for Basic Biology, Japan
- Lecturers:
  Dr. Tomonori DEGUCHI (AIST)
  Dr. Rie GOTO (Ehime Univ.)
  Dr. Masayuki HATTORI (NIBB)
  Dr. Shinya KOMOTO (EMBL)
  Dr. Akiko KONDOW (Fujita Health Univ.)
  Dr. Laszlo ORBAN (TLL)
  Dr. Sachihiro SUZUKI (OIST)
  Dr. Saori YOKOI (NIBB)
  Dr. Goro YOSHIZAKI (TUMSAT)
  Dr. Zoltán VARGA (Zebrafish International Resource Center/ Univ. of Oregon)
- Course Staff:
  Dr. Yoriko ANDO (NIBB), Dr. Satoshi ANSAI (NIG), Dr. Shoji FUKAMACHI (Japan Women's Univ.), Dr. Akiko KONDOW (Fujita Health Univ.), Dr. Shin-ichi HIGASHIJIMA (NIBB), Dr. Yasuhiro KAMEI (NIBB), Dr. Shinya KOMOTO (EMBL), Dr. Kiyoshi NARUSE (NIBB), Dr. Shigenori NONAKA (NIBB), Dr. Sachihiro SUZUKI (OIST), Dr. Yusuke TAKEHANA (NIBB), Dr. Saori YOKOI (NIBB)
- Contents of the course:
  Gene knock-in using the CRISPR/Cas9 system in zebrafish, Gene knock-out using the CRISPR/Cas9 system in medaka, Highly efficient transgenesis using the PhiC31 system in medaka, Optomotor response in medaka mutant, Local gene induction with infrared laser-evoked gene operator (IR-LEGO) in medaka, in vivo imaging, Cryopreservation of sperm and artificial insemination in medaka

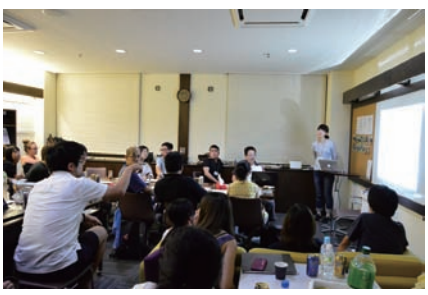


The 9th NIBB International Practical Course - 4th NIBB-TLL Joint International Practical Course was held from August 18 to 30, 2016. We received 28 applications, and 17 (8 males, 9 females) passed the screening process. The participants comprised a group of 7 from Japan and 10 from overseas of 10 regions in 7 countries. Dr. Laszlo Orban, one of the organizers of the program, attended as a special lecturer as well, and we invited other researchers to give special lectures during the course such as Dr. Zoltan Varga, who is the director of the Zebrafish International Resource Center, the University of Oregon, Dr. Rie Goto from Ehime University, and Dr. Goro YOSHIZAKI from Tokyo University of Marine Science and Technology. Moreover we invited 7 lecturers from both Japan and abroad to give seminars. The course was covered the topics "Highly efficient transgenesis using the PhiC31 system", "Gene knock-in using the CRISPR/Cas9 system in zebrafish as well as medaka", "Cryopreservation of sperm and artificial insemination in medaka", "Imaging using light-sheet microscopes", and "Gene expression induction method by IR-LEGO".

We managed the course without supporting the travel costs for students, however in spite of having supported travel costs in the past we had just under twice as many applicants for this year's course. It was the first time we hosted participants from South America, of which we had two. We were also pleased that we received students from both EMBL and TLL, two of NIBB's research cooperation institutes.

We plan to continue to conduct NIBB International Practical Courses which feature small fish research with a combination of basic laboratory techniques and the latest technology in the future.

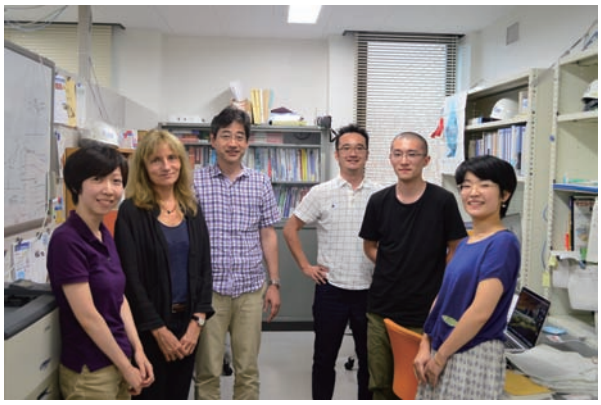
(Kiyoshi Naruse on behalf of the organizing committee)

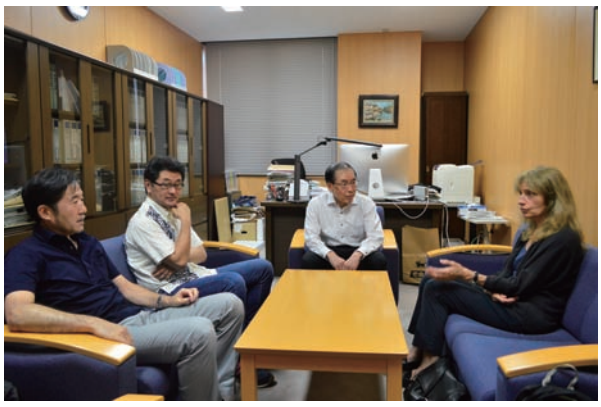


NIBB-EMBL Collaboration
Dr. Anne Ephrussi's visit to NIBB

Dr. Anne Ephrussi, the head of the Developmental Biology Unit of EMBL, visited on July 12nd, 2016. She gave a seminar titled "An atypical RNA-binding Tropomyosin recruits kinesin-1 to oskar mRNA for transport in the *Drosophila* oocyte" and visited the Laboratory of Neuronal Cell Biology to have a discussion with the members of Prof. Shiina's team about intracellular RNA transport.

In addition, Dr. Ephrussi had a meeting with Dr. Masayuki Yamamoto, Director General of the National Institute for Basic Biology, and Prof. Naoto Ueno on the future of collaborative activities between EMBL and NIBB.





NIBB-Princeton University Collaboration
Prof. Ileana M. Cristea's visit to NIBB

Prof. Ileana M. Cristea from the Department of Molecular Biology, Princeton University, visited on September 23rd, 2016. Prof. Cristea has been conducting an international joint research project, financially supported by NINS, with the Division of Morphogenesis at NIBB. She gave a seminar titled "Virology meets Proteomics: Understanding human organelle remodeling in space and time during viral infection" exclusively for NIBB's researchers, and had a meeting with the members of the Division of Morphogenesis, Prof. Ueno's group, to discuss the future of the joint research project.

The 10th NIBB Bioimaging Forum "Pioneering Bioimaging in the New Era"

Organizer: Yasuhiro Kamei, Hideki Takami, Yutaka Hayano, Mitsuo Takeda, Hirotsugu Yamamoto, Masayuki Hattori, Takashi Murata, Shigenori Nonaka, Yosuke Tamada

We held the 10th NIBB Bioimaging Forum on February 16th and 17th 2016. The subtitle for this meeting was "Pioneering Bioimaging in the New Era", and aimed at the development of new fields in bio-imaging by asking experts in various disciplines (optics, engineering, mathematics, astronomy, biology) to give lectures on new and anticipated imaging methods on the horizon. The forum was divided into 5 sessions; on the first day "The Present State and Problems of Imaging in the Fields of Biology and Astronomy" was addressed, followed by "Optical Theory Opening Up the Future of Imaging", which covered the latest optical theory on holography and coherence, and the lecture "Creation of New Imaging Technology 1" on topics such as tomography was given. On the second day, we held the lecture "State-of-the-Art Image-Sensors and Devices" on cutting-edge camera technology and "Creation of new Imaging Technology 2" on the sparse modeling theory. There were 52 people in the audience, and more than 10 industry attendees. At the end of the forum a comprehensive discussion was held with all members, including the audience, and a consensus was reached on the necessity and difficulties of fusing the imaging fields. In addition, nearly 30 participants remained in the venue after the forum, and enthusiastic discussions and consultations on joint research took place. Although meetings aiming at establishing new bioimaging methods through fusion of different fields are uncommon in Japan the active interaction of participants at this forum lead to its success.

(Yasuhiro Kamei)

The NIBB Genome Informatics Training Course

The NIBB Core Research Facilities organizes a series of training courses on up-to-date research techniques. The NIBB Genome Informatics Training Course (GITC) is specially designed for biologists who are not familiar with bioinformatics. In 2016, we held two sets of training courses on RNA-seq analysis and one course on BLAST analysis. While we previously provided a 3-day program on RNA-seq analysis, we rearranged the course this year dividing it into two 2-day programs: one was a preparatory course to learn basics of UNIX and R and the other was a practical course to learn pipelines of RNA-seq analysis using next-generation sequence data. The new BLAST analysis course aimed to enable participants to get familiar with a large scale sequence database search in the local environment using BLAST software for systematic sequence data analyses. These GITC courses offered lectures and hands-on tutorials.

Introduction to RNA-seq – from the basics of NGS to *de novo* analyses

February 25(Thu)-26(Fri), 2016

(Preparatory Course) Basics of UNIX and R

- Organizer: Dr. Ikuo Uchiyama (NIBB Core Research Facilities)
- Lecturers: Dr. Ikuo Uchiyama, Dr. Shuji Shigenobu, Mr. Tomoki Miwa, Ms. Hiroyo Nishide, Mr. Takanori Nakamura (NIBB Core Research Facilities)
- Participants: 22
- Program:
  1. UNIX for beginners
  2. Introduction to "R"
  3. Text processing
  4. Shell scripting
  5. Exercises

March 10(Thu)-11(Fri), 2016

(Practical Course) RNA-seq analysis pipeline

- Organizer: Dr. Shuji Shigenobu (NIBB Core Research Facilities)
- Lecturers: Dr. Shuji Shigenobu, Dr. Ikuo Uchiyama, Dr. Katsushi Yamaguchi, Ms. Hiroyo Nishide (NIBB Core Research Facilities), Dr. Masanao Sato (Keio Univ.)
- Participants: 21 (including 1 from NIBB)
- Program:
  1. NGS basic data formats and NGS basic tools
  2. Introduction to statistics
  3. RNA-seq pipeline: genome-based and transcriptome-based approaches
  4. Multivariate statistics
  5. Exercises

Introduction to RNA-seq – from the basics of NGS to *de novo* analyses

August 25(Thu)-26(Fri), 2016

(Preparatory Course) Basics of UNIX, R, and NGS

- Organizer: Dr. Shuji Shigenobu, Dr. Ikuo Uchiyama (NIBB Core Research Facilities)
- Lecturers: Dr. Shuji Shigenobu, Dr. Ikuo Uchiyama, Mr. Tomoki Miwa, Dr. Katsushi Yamaguchi, Ms. Hiroyo Nishide, Mr. Takanori Nakamura (NIBB Core Research Facilities), Dr. Masanao Sato (Hokkaido Univ.)
- Participants: 22 (including 3 from NIBB)
- Program:
  1. UNIX for beginners
  2. Introduction to "R"
  3. NGS basic data formats and NGS basic tools
  4. Text processing in UNIX
  5. Introduction to bias4 system
  6. Exercises

September 8(Thu)-9(Fri), 2016

(Practical Course) RNA-seq analysis pipeline

- Participants: 22 (including 3 from NIBB)
- Program:
  1. Review NGS basic data formats and NGS basic tools
  2. Introduction to statistics
  3. RNA-seq pipeline: genome-based and transcriptome-based approaches
  4. Multivariate statistics
  5. Exercises

Mastering BLAST, the essence of sequence analyses

December 1(Thu), 2016

- Organizers & Lecturers: Dr. Shuji Shigenobu, Dr. Ikuo Uchiyama (NIBB Core Research Facilities)
- Participants: 20 (including 4 from NIBB)
- Program:
  1. BLAST for beginners
  2. Local BLAST search with command-line
  3. BLAST inside
  4. Large scale BLAST search
  5. Annotation of genes and ortholog analysis
  6. Beyond BLAST

The 4th BioImaging Analysis Training Course

Organizers: Dr. Kagayaki Kato, Dr. Yoshitaka Kimori, Dr. Yasuhiro Kamei, Dr. Hiroshi Koyama, Dr. Shigenori Nonaka, Dr. Takashi Murata

December 5 (Mon) -7 (Wed), 2016

The 4th Bioimaging Training Course was held jointly by the Center for Novel Science Initiatives' Department of Imaging Science, JSPS KAKENHI Platforms for Advanced Bioimaging Support (ABiS) and NIBB. This course was designed for biologists who are relatively new to analyzing datum obtained through advanced microscopy. Therefore the focus of training was learning image processing and analytical techniques through "solving simple problems with image analysis" and "understanding appropriate methods and necessary preparation for consulting experts in technically advanced imaging challenges". 58 people applied for the course, which had a maximum capacity of 21 participants, this clearly suggests the height of the demand for courses on these subjects.

This course's lectures were conducted with the aim of training participants to keep in mind the series of steps essential to fundamental image processing and analysis while obtaining images to be used (workflows). In addition, we loaned the participants PCs pre-installed with ImageJ, a typical open-source software package for biological image processing and analysis, and images which were used for practicing the basic operations and settings of image processing. Also, lectures were given on how programming of simple "macro-programs" which use these workflows in ImageJ allows automation, which is essential for the large capacity and high-dimensional throughput of microscopic imaging which has become common in recent years.

At the conclusion of the course each of the students gave commentary and discussed the methods used with examples of actual images from their own research. Every year after the course, participants express feeling "pretty tired, and satisfied" as part of the questionnaire, and certainly there is a true benefit in terms of their image analysis by becoming more familiar with these techniques. In addition, we expect that this course will increase opportunities for joint research relating to biological image analysis.

(Kagayaki Kato)

The NIBB Internship program

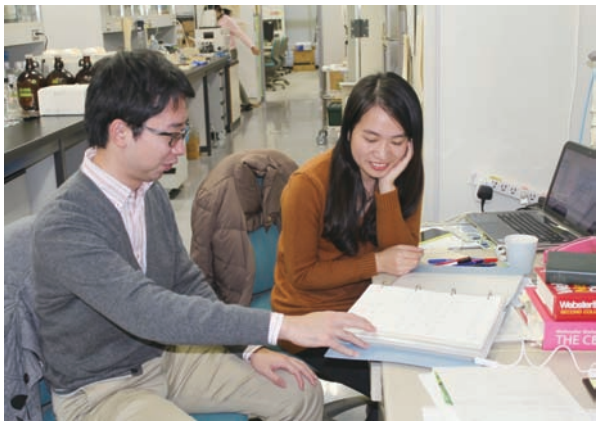
The NIBB Internship program, started in 2009, is a hands-on learning course for overseas students designed to give high-quality experience in real world research and focused education of biology. At the same time, this program aims to internationalize the graduate students of SOKENDAI (Graduate University for Advanced Studies), giving them the opportunity to get to know students and interns with various cultural customs. Another goal of the program is to build connections through providing education to the people who will form the core of international research networks in the future.

To participate in this program, applicants who would like to experience research at NIBB must supply the name of the lab they would like to visit as well as their reasons for choosing it, and a letter of recommendation. Based on this information applicants are chosen to spend set periods of time participating in specific research activities in the lab they applied for. Round trip airfare and housing expenses are provided by the NIBB Internship Program.

In FY 2016 there were 23 applicants, out of which five interns were selected. These interns were from universities located in 5 countries (India, Germany, Turkey, USA, and Vietnam) and spent periods ranging from four to twelve weeks experiencing life as a member of a research team.

Report from a participant
Tran Thi Hong Nguyen
VNU University of Science, Vietnam

I am Tran T.H. Nguyen, from Hanoi, Vietnam. I have just completed my Bachelor's in Microbiology from Vietnam National University, University of Science. I was so glad to be an NIBB internship student under the guidance of Professor Takada from 10th Oct to 28th Dec, 2016. During my internship in the molecular and developmental laboratory, I focused on the expression of EGFP fluorescently tagged Wnt3a protein during the embryogenesis of Xenopus embryos.



In my research, I planned to visualize Wnt proteins by the addition of fluorescent tags because it is known to be quite difficult to generate anti-Wnt antibodies available for immunohistochemistry. Prior to visualization of tagged-Wnt proteins in embryos, in the beginning, I focused on the optimization of linker length to minimize the effect of tags on the activities of Wnt proteins. According to some recent research, it was shown that activities of Wnt proteins are frequently damaged by the addition of fluorescent tags. Therefore, I tried to optimize the design of tagged proteins by changing the length of the liker peptide connecting Wnt to EGFP tag. Specifically, I generated 5 constructs in which the lengths varied from 9 to 55 amino acids. These constructs were expressed in culture cells and in Xenopus embryos and examined to what extent Wnt3a activity and EGFP fluorescence were retained.

During my three months of stay in Okazaki, I had lots of unforgettable memories with labmates and other friends here. I was able to learn some interesting research topics in my internship under the valuable guidance and inspiration of Prof. Takada, my mentors – Ritsuko san, Mii san, and the warm hearts of Nobata-san, Utsumi-san, Takashiro-san and other labmates who encouraged me to successfully complete my internship here. During my first visit in Japan, my flight was delayed by 2 hours and I arrived in Nagoya at midnight. It was so touching that Prof. Takada and his wife came to receive me from the airport so late at night.

The NIBB internship program is really meaningful for international students who want an experience with one of the highest reputations in education as well as the Japanese culture. Thanks all, for everything I experienced here!

INDEX

INDEX

INDEX

Access

From Tokyo

Take the Tokaido Shinkansen and get off at Toyohashi, change to the Meitetsu Line and get off at Higashi Okazaki Station (approximately 20 minutes from Toyohashi).

From Osaka

Take the Tokaido Shinkansen and get off at Nagoya, change to the Meitetsu Line and get off at Higashi Okazaki Station (approximately 30 minutes from Nagoya).

From Central Japan International Airport

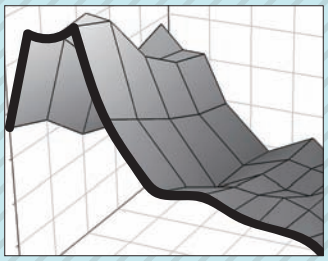
Get on the Meitetsu bus bound for JR Okazaki Station and get off at Higashi Okazaki Station (approximately one hour from the airport). Alternatively, take the Meitetsu Line for Nagoya and change at Jingu-mae Station to a Toyohashi-bound train and get off at Higashi Okazaki Station (approximately 70 minutes from the airport).

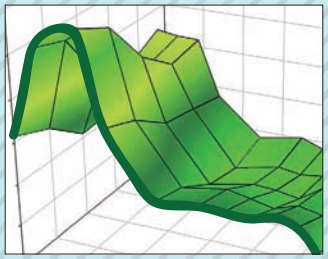
From Higashi Okazaki Station to Each Area

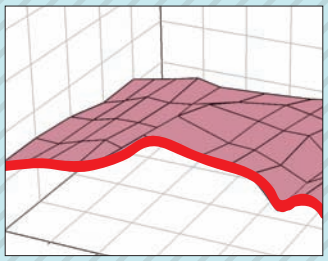
Turn left (south) at the ticket barrier and exit the station. The Institute is a 7-minute walk up the hill (Myodaiji-area) or 20-minute walk (Yamate-area).

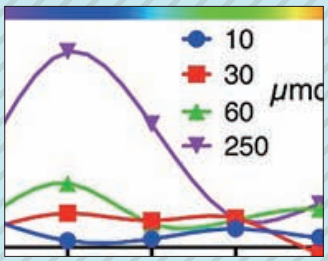
By car

Take the Tomei Expressway to the Okazaki Exit. Turn left at the City Hall S. signal (approximately 10 minutes from the Exit).









National Institutes of Natural Sciences
National Institute for Basic Biology

38 Nishigonaka, Myodaiji, Okazaki, Aichi 444-8585 Japan
tel: +81 564-55-7000 fax: +81 564-53-7400
http://www.nibb.ac.jp/en/
Issued in December 2017
Edited by the Research Enhancement Strategy Office, NIBB