Technology with High-Speed Movies to Analyze the Movement of Internal Organs in Medaka

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Living organisms are always exposed and respond to various stresses from the external world – the environment, such as temperature, gravity, radiation or circadian. Understanding and addressing the effects of the environment on human health is a major issue. To clarify this issue, we are studying both human and medaka. Medaka is a good model organism to verify the effects of the space environment for their features including their short life cycle, productivity, mapped whole genome sequences, and transparency even in adults. We are trying to evaluate the heartbeat using transparent medaka with live imaging. Despite the importance of functions of internal organs, its observation has been difficult. Imaging technologies has developed remarkably in a decade. With the retirement of the space shuttle, experimental procedures without sample collection will be required.

We established an approach to acquire the images and the methodology of the analysis from high-speed movies. We are subsequently going to introduce a method of verifying the movement of internal organs in medaka experiments using high-speed movies.

Key Words: Medaka, Live Imaging, Space Biomedicine

1. Introduction

The full-scale International Space Station (ISS) operation has begun. With the astronauts staying in space, it is important to keep their activity there healthy, safe and comfortable. Understanding the influences of environmental stress on astronauts during space flight is a major focus of space medicine. Its purpose is to reduce the influences of the space environment and improve performance associated with an astronaut's extended stay in space. To achieve this, Japan Aerospace Exploration Agency (JAXA) Space Biomedical Research Office (J-SBRO) dedicates itself to understanding and reducing the influences of the space environment on human health by establishing a method to resolve and clarify the mechanisms of those medical problems¹⁾. Under such circumstances, we are trying to understand and address the effects of the space environment on human health. Mutual basic and clinical research is important in generating a synergistic effect in space medicine or in fields related to human health. Our goal is thus to reduce the risk of influences of the space environment efficiently and safely by using model organisms – medaka, *Oryzias latipes*.

Medaka, small teleost and native to Japan, South Korea, Taiwan and the North-East of China, which lives in fresh water, has been established as a model organism²⁾. They are highly adaptive to a wide temperature range and low O₂ content. Recently, small fish like medaka and zebrafish have been used as disease models³⁻⁹⁾. JAXA is developing an

aquatic habitat (AQH) system, which enables us to rear and keep small fish on the ISS for up to 3 months. There are plans to commence the medaka experiment on orbit in 2011¹⁰⁾. It is important to resolve the stress response at the molecular, cellular, tissue and individual level analyses, which have been progressing in recent years, and clarify changes in living organisms. With their transparent bodies through the development and even adults in some strains, medaka have helped clarify mechanisms from live imaging¹¹⁻¹³⁾. This transparency is a key feature when evaluating the phenomena via live imaging.

Recently, technology concerning imaging has developed remarkably. There are numerous techniques in microscopy, applications, cameras and communication technologies using satellites. The diversification of tools like fluorescent compounds contributes significantly to measuring enzyme activities, such as lipid metabolism at both cellular level and in vivo¹⁴⁻¹⁶⁾. Following the retirement of the space shuttle, material transportation between the ISS and Earth must rely on rocket vehicles, hence experimental procedures without sample collection will be required in the near future. Live imaging could be one of the solutions. Observation will be more general, not only due to the limitation of sample return but also the potential of the non-invasive approach.

We are thus planning several experiments using live imaging techniques and preparing the ground study. We could develop a method for heart rate analysis from high speed movies and evaluate the alteration of the autonomic nervous

system through the same. We will report the establishment of the analytical method from the movies.

2. Materials and Methods

2.1. Animal husbandry

Medaka (*Oryzias latipes*) strains were obtained from our breeding colony and analyzed more than 3 months as adults. Fish were kept under standard laboratory conditions in a 26°C incubator with a 14:10-h light-dark circadian cycle. All adult experiments were performed with average sized fish, reared in common tanks and from the same clutches. Each experiment was conducted within 5 minutes. The animal protocols were approved by the Committee of Institutional Animal Care.

2.2. Imaging system and digital video settings

Cardiac activity was monitored using digital high-speed videomicroscopy, using an inverted stereo microscope connected to a digital high-speed camera, (Exilim Ex-F1, Casio, Tokyo, Japan). A camera capturing video at the 300 frames s⁻¹ with 512x384 pixels was used to capture videos of the internal organs and recordings were performed with 1.25X objectives.

2.3. Heart rate variability analysis

The heartbeat data was obtained from the peak of the intensity of each heartbeat. The time series of the interbeat interval (IBI) were first detrended and then subdivided into 128-point segments. Data were linearly interpolated and resampled at 4 Hz to create an equidistant time series for spectral analyses. The analysis was implemented with each Hanning-windowed data segment and then averaged to calculate the IBI autospectra. The minimal resolution of these spectra is 0.03125 Hz. This data analysis was performed using DADiSP software (DSP Development, Cambridge, MA, USA).

3. Results and Discussion

3.1. Selection of Medaka strains

To establish the live imaging experiment, we had to decide the species based on several points – ease of handling and transparency of the internal organs. We selected 2 incross medaka lines for our experiments, T5 and SK2 (Fig. 1). The T5 strain has recessive alleles of the 5 loci, *b* as amelanotic melanophores, *gu* as abnormal iridophores, *lf* as no leucophores, *i* as complete albino phenotype, and *wl* as colorless leucophores and *b* alleles in the *i* locus. The SK2 strain is homozygous for 3 recessive pigmentation mutations (*bg8*, *lf*, *gu*) derived from a southern Japanese population of wild-type medaka^{17, 18}). The SK2 strain is suitable for the observation of almost all kinds of internal organs, including the liver or spleen. The T5 strain has melanophores in the eyes and peritoneal membranes so that only heart can be the target for the live imaging, and has good eyesight meanwhile SK2 lost eyesight without melanophore. No significant difference was observed in the cardiac activity of the two strains (data not shown).

This result suggested that see-through medaka or some strains of medaka with transparency, even in adults can be

good models for the evaluation sample using live imaging. Good adaptability of medaka to various environments also has advantages, especially in terms of transportation to space, so as well as the transparency of its internal organs, other characteristics also make medaka an excellent experimental animal for space biomedicine.

Fig. 1. Selected two medaka strains. SK2 has a transparent peritoneal membrane and enables the observation of internal organs, such as the heart, liver and gut (A). T5 strain has a black peritoneal membrane but the heart is visible due to the lack of iridophores in this strain (B). e: eye, f: fin, gi: gills, gu: gut, h: heart, li: liver, p: peritoneal membrane.

3.2. Imaging system and digital video recording

To reduce the load or stresses on the medaka, we modified the data acquisition system (Fig. 2). The heart of the medaka is visible from the ventral side of the fish, hence the stereomicroscope for observation and taking images is inverted instead of flipping the medaka. A mold is made with banks in the Petri dish, into which fish were placed under partially immobilized condition in each experiment, and maintained at 26°C during the experiment. Digital high speed movies lasting a maximum of five minutes were captured and recorded on a computer using Final Cut Pro software (Apple Inc., CA, USA) for image processing.

Fig. 2. Schema of the analysis system. The images were taken by a high-speed camera attached to the inverted microscopy, which requires LED light. Samples were placed in a special mold with netty banks, which is softly restrained to reduce the load of the samples themselves. Images were taken using a high speed camera and processed for analysis with the program for the operational algorithm.

Although many kinds of methods were used to detect the movement of internal organs such as electrocardiogram recordings for heartbeat using adult fish¹⁹), the treatment

involved in placing the electrode exerts a considerable load on the fish. Moreover, it is highly difficult to manipulate these procedures for small fish like medaka. Recently the imaging technique is becoming more familiar for fish study, especially in embryos or fry²⁰⁾. Ultrasonography or contrast medium are also used for the observation analysis of internal organs due to the importance of their functions²¹⁾. This result showed how live images enable movement inside the body to be detected, even in the adult fish.

3.3. Extraction of cardiac activity from images

To analyze the cardiac activity, images of the heart beating were taken at 300 frames·s⁻¹ and stored on the on-board memory. Three-minute stored image sequences (54,000 pictures) were subsequently analyzed, mainly by the intensity of the heart region by means of image-processing and analysis software Bohboh 4.02 (Bohbohsoft, Tokyo, Japan) (Fig. 3). Boxes of equivalent shape and size were drawn on the medaka body, blue for heart region and green for the body region (Fig. 3A), the intensity of which was translated into numeric data and stored in a text file for all image frames of the whole image sequence. The intensity profile was analyzed for local maxima and minima to identify the end systole and diastole (Figs. 3B and C, respectively).

Fig. 3. Extraction of the cardiac activity from the image. The selected areas shown in blue boxes were placed to calculate the intensity of the heart region and green box was to the intensity of the body region (A). The sample image of the heart and box at the timing of diastole (B) and systole (C). D-F: 5 seconds examples of the heartbeat extraction. D: The average of intensities luminance in the heart region (blue). Intensities have two different peaks in one heartbeat. Luminance shows the total intensity of the region. E: The intensity luminance in the body region (green). Each peak shows one aspiration caused by gill movement. F: The subtracted luminance body region intensity from the heart region followed by 21 points smoothing algorithm (black). To remove the body movement properly from the heart region intensity, each experiment requires each variable. This line shows the heart movement. a: air bladder, e: eye, g: gills, h: heart, m: mold, p: peritoneal membrane.

The text file was imported into a computer program using mathematical software Cutwin 8.10 (Ever Green Soft, Tokyo, Japan). A smoothing algorithm averaging among 21 points was used to correct the small number of misidentified peaks and peak detection errors. During the detection of the intensity alterations, two different movements seemed to be included in the heart region (Fig. 3D). As we tried to define the two different movements, we finally found that they were the heartbeat and body movement derived from aspiration (Fig. 3E). The subtracted signals of intensity in aspiration followed by smoothing were defined as the event of the beating heart (Fig. 3F).

These results suggested that the specific algorithm we established in this experiment enables the requisite information to be extracted even from images including unnecessary movements, such as body movements with aspiration.

3.4. Quantification of cardiac activity

A count of the minima as diastole divided by the length of the movie in minutes provides a measure of the heart rate. The time between minima gives a measure of the interbeat interval, as shown in Fig. 4A. The heart rate altered over time. Here we show one example of the FFT analysis in Fig. 4B. This data showed that two peaks appeared in the heart rate variability. Peaks are indicated as fluctuations of the heart rate variability, which are supposedly derived from the autonomic nervous system. In this case, the fluctuation in the heart rate showed specific peaks at 0.6 and 1.0 Hz.

Fig. 4. Example heart rate analysis using DADiSP software. (A): The heart rate alteration over 24 seconds. The fluctuations in the heart rate occurs during this sampling time. (B) FFT analysis. In this case, the fluctuation in the heart rate has specific peaks at 0.6 and 1.0 Hz.

We have established a system in which it is possible to observe changes in cardiac function in the unanesthetized medaka. The goal of this study has been to develop an assay to measure change in internal organs, such as the cardiac

function, in response to environmental change and determine the mechanisms of these regulatory systems.

4. Conclusion and Perspectives

In this report, we claimed to have established an analytical system to take image data, extract essential information and analyze the extracted data. We also decided on the use of transparent medaka as the sample in this experiment. As we explained above, following the retirement of the space shuttle, it is important that experimental procedures be conducted without sample collection as live imaging with small animals – medaka.

The aim of basic research using model organisms is to understand the biological impacts under space environment for the healthcare of astronauts. However, this research is also for people living on Earth to promote healthy lives such as improving living environments or drug screening.

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