SIP Protocol Series No.3

A rapid method to analyze meiofaunal assemblages using an Imaging Flow Cytometer

Cross-ministerial Strategic Innovation Promotion Program

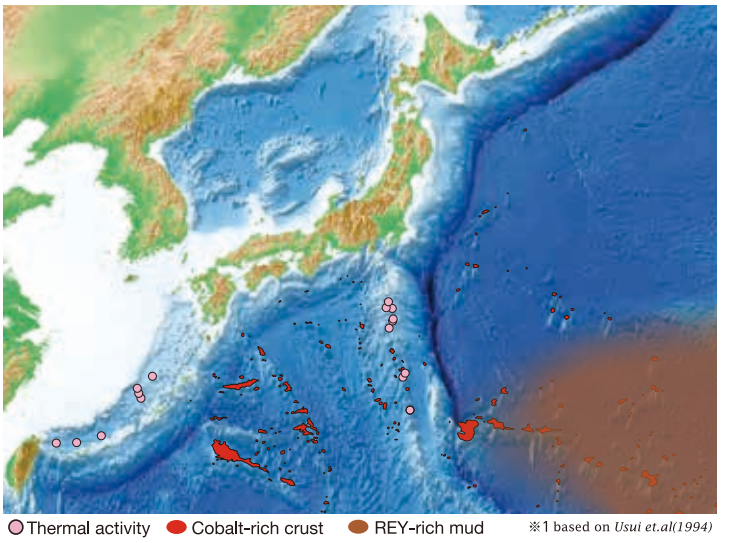
The Strategic Innovation Promotion Program (SIP) was launched by the Council for Science, Technology, and Innovation (CSTI), which oversees projects that target scientific and technological innovation in line with Japanese government directions as stated in the Comprehensive Strategy on Science Technology and Innovation and the Japan Revitalization Strategy. This interdisciplinary program among government agencies, academic institutes and private sectors addresses eleven issues. One of these issues is Next-Generation Technology for Ocean Resources Exploration.

Zipangu in the Ocean Program and Protocols for Environmental Survey Technologies

Zipangu in the Ocean Program is a technical study of the development of submarine mineral deposits that takes into consideration the wise use of these resources.

One research area is the ecological survey of organisms and their long-term monitoring. However, an ecosystem consists of various interrelated factors; thus, in addition to a comprehensive understanding of the system, observation and analysis of each component to its most elemental level are unavoidable. Recently, increased environmental awareness and the necessity of forming a consensus have become key issues in conducting development activities. Growing concern for the environment by the public and the diversification of the use of maritime areas have complicated the interests of stakeholders. To facilitate the formation of a consensus under these conditions, it is important for standardized methods to be implemented. This will ensure that research processes are transparent and that the collection of survey data is objective.

This protocol series aims to introduce more accurate, user-friendly, objective and effective underlying technologies required to understand the environmental impact of submarine mineral resource development. We believe that creating such a technology tool-kit will allow us to develop these resources in a sustainable manner.



※1 based on *Usui et.al(1994)*

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chapter 1

Preface

1-1. Current status of meiofauna analysis

Meiofauna are usually defined as benthic organisms that pass through a 500–1,000 μm sieve and are retained on a 32–63 μm sieve[*1] (Giere 2009). Compared to larger macrofauna and megafauna, meiofauna in the deep sea have high abundance and biomass and are an important component of deep-sea ecosystems (Wei et al., 2010). In addition, meiofauna have considerable influence on the nutrient cycling in the sediments and sediment stability. Therefore, meiofauna are important as biological indicators used to monitor natural or anthropogenic disturbances (Zeppilli et al., 2015). Indeed, even in the International Seabed Authority (ISA) guidelines for contractors on the assessment of possible environmental impacts due to exploration activities (ISA, ISBA/19/LTC/8) mandate the reporting of the abundance and diversity of seafloor biotic communities, including meiofauna and the effect on keystone species. This is as per the Environmental Impact Assessment (EIA) necessitated by activities related to the exploration of seafloor mineral resources. In other words, the ISA, which is responsible for the management of deep sea mineral resources, seeks EIAs for meiofauna. However, traditional methods for meiofauna community analysis are extremely time-consuming, which is economically problematic due to the costs of conducting EIA as part of resource development. In addition, advanced expertise is required for the identification of meiofauna to the species, genus, or even family level, and the number of qualified to do this is limited. Also, if a technician does not have the training or knowledge to identify meiofauna, the dissemination of inaccurate data could result. For these reasons, accurate, efficient and objective analytical tools for identification of meiofauna are needed.

1-2. Analytical methods for other organisms

Fundamentally, the most reliable method of investigating biotic communities is to collect organisms themselves and have them identified visually by trained specialists. However, in recent years, concurrent with the development of camera technology, video and image-based techniques for mega- and macrofauna community analysis have been adopted (Durden et al., 2016). Also, in EIA study, change in the megabenthos community related to the drilling activity was analyzed based on images captured by submersibles (Nakajima et al., 2015).

Various devices have been also developed to obtain images of plankton in the water column so that these organisms can be analyzed (e.g., Visual Plankton Recorder [VPR], ZooSCAN, and FlowCAM). Imaging flow cytometers that automatically obtain images of plankton and other small size particles in fluids such as seawater can also be considered for use in meiofauna research.

1-3. Determination of level of classification to be analyzed

To identify to the species level based on morphological information and analyze community composition is extremely difficult for meiofauna study. For example, in waters that have not been surveyed, more than 95% of benthic copepods are considered undescribed species (Seifried, 2004). In addition, there are reports that species that appear to have quite similar morphologies have been confirmed by molecular biological techniques to be separate species (Schizas et al., 1999; Rocha-Olivares et al., 2001; Easton et al., 2010).

On the other hand, the concept of taxonomic sufficiency (TS) in which higher classifications than at the species level (such as genus, family, order) without loss of information has been suggested as a method when evaluation of the environmental impact of anthropogenic disturbances (Ellis, 1985). Most species are adapted to a narrow range of natural gradients and unable to adapt to anthropogenic disturbances (Warwick, 1988a, b; Bacci et al., 2009). That is, natural environmental gradients such as water depth and sediment chemical characteristics lead to changes in community composition at the species level, while anthropogenic disturbances lead to changes in community copmosition at the higher taxonomic levels (Warwick, 1988a, b).

1-4. Scope of this manuscript

Based on the above issues, this paper will provide information with the goals of:

- obtaining information on community composition at a higher taxonomic level than species,
- obtaining image data rapidly,
- analyzing the meiofauna community,

and imaging flow cytometry will be introduced as an analytical method (Kitahashi et al., submitted).

*1
However, ISA guidelines (ISBA/19/LTC/8) define meiofauna as >32 μm and <250 μm in size.

chapter 2

Field work

2-1. Sample collection

For analysis of meiofauna community composition and diversity, we should quantitatively collect marine sediments. Examples of equipment (corers) for quantitative sampling include box corers, grab corers, and multiple corers (Barnett et al., 1984). Since meiofauna is concentrated in the surfaces of sediments (Kitahashi et al., 2014, and others), it is preferable to use a multiple corer for meiofauna research as it can obtain samples without disturbing the sediment surface. The multiple corer has the advantage of being able to collect several sediment cores at once. Thus, for a single survey site, in addition to the methods introduced by this paper, sediment samples can be subjected to traditional microscopy observations, metagenomic analysis, chemical composition analysis and sediment analysis.

Corers mentioned above are deployed from a research ship to the seafloor; thus, it is not suitable for surveys that require selection of sampling points while observing seafloor conditions. In areas where pin-point sampling is required, e.g., hydrothermal fields where chimneys and mounds exist, sampling using push corers operated by manned or unmanned submersibles is recommended.

In addition, it has been reported that if different samplers or corers with different diameters are used, community composition and vertical distribution of meiofauna can be changed (Sampler Bias, Bett et al., 1994; Shirayama and Fukushima, 1995; Kitahashi et al., 2010). Thus, for the same EIA program, identical samplers should be employed, and when comparing results with other programs, differences in sampling gear must also be considered.

2-2. Fixation and storage of samples

The vertical distribution pattern of meiofauna in sediment layers is important information for the EIA; therefore, it is necessary to slice layers from the sediment surface downwards. Each sediment layer should be fixed and preserved separately.

1 Equipment and reagents

- Spatulas
- 0.22 μm filters
- Siphon tubes
- Plastic bags
- Wash bottles
- Pipettes
- Extruder
- Neutral buffered formalin
- Seawater

2 Procedure

1. Make filtered seawater using a 0.22 μm filter and keep it in a wash bottle.
2. Place the collected sediment core onto an extruder.
3. Using a siphon tube, remove seawater above the sediment to approximately 1 cm above the sediment surface.
4. Using a syringe, remove the remaining seawater 1 cm above the sediment and place in a plastic bag.
5. Using a spatula, slice the sediment cores into layers: 0–0.5, 0.5–1, 1–2, 2–3, 3–4, and 4–5 cm from the surface. Place each layer into separate plastic bags.
6. Use filtered seawater to rinse off any sediments remaining on the spatula into the same plastic bag holding the layer.
7. Use tap water to completely wash the spatula and dry it thoroughly with Kimwipes or the like each time the spatula is used.
8. Add 100% neutral buffered formalin to the samples for a final concentration of about 5%.
9. Firmly seal the plastic bag and store.

3 Notes

Fixation with formalin is optimal for preservation of the morphology of meiofauna. However, if further analysis is to be conducted after the initial observation, it is possible to fix and store the samples by methods such as freezing or adding ethanol, Lugol' s solution, glutaraldehyde, DESS (Yoder et al., 2006) or RNAlater (Invitrogen).

chapter 3

Analysis using imaging flow cytometer

3-1. Pre-processing

Many of the applications for imaging flow cytometers are for imaging small particles floating in fluids such as water. Thus, when attempting to observe meiofauna living in sediments, several issues arise: 1) sediment samples without pretreatment blocked the narrow flow cell, preventing observations, 2) sediment particles are overwhelmingly present in greater numbers compared to meiofauna and image data analysis is time-consuming if sediment samples are introduced to the imaging flow cytometer as is, 3) many images must be acquired for the abundant sediment grains, which increases the data volume. For these reasons, 4) obtaining images of target meiofauna is complicated.

Based on differences in density of meiofauna specimens and sediment particles, a centrifugal separation method using colloidal silica solution has been devised (Burgess, 2001; Danovaro, 2010). This method was applied and subsequently, in our case, enabled us to observe meiofauna using an imaging flow cytometer (Kitahashi et al., submitted; JP Patent Application 2016–232868).

1 Equipment and reagents

Sieves
(mesh sizes: 1 mm, 250 µm, 63 µm)
Spoons
Wash bottles
50 mL conical tubes
Tube stands
Centrifuge
Tap water
Rose bengal
Colloidal silica solution
(Ludox HS-40, Sigma-Aldrich, Inc.)

2 Procedure

1. Add rose bengal solution to the sediment samples (final concentration, 0.05 g/L) and leave to stand for at least one night.
2. Sequentially pass the sediment samples through 1 mm, 250 µm and 63 µm mesh size sieves. Rinse away the seawater using tap water as much as possible.
3. Using tap water, transfer fractions retained on the 1 mm and 250 µm mesh size sieves to 50 mL conical tubes. Add formalin for storage.
4. Use a spoon to transfer the fraction retained on the 63 µm mesh size sieve to a 50 mL conical tube. Keep the tube as dry as possible.
5. Rinse any sediments remaining on the sieve into a conical tube using a wash bottle containing Ludox HS-40.
6. Centrifuge the conical tube containing sediment/Ludox HS-40 at 800 × g for 10 min.
7. Transfer the supernatant to the sieve.
8. Add more Ludox HS-40 to the pellet, resuspend, and centrifuge again; repeat once more for a total of three times.

9. Using Ludox HS-40, transfer particles retained on the sieve to a conical tube.

3-2. Observations using imaging flow cytometer

FlowCAM(Sieracki,1998; Figure 1) is introduced here as an example of an observation using an imaging flow cytometer. Operation procedures can differ depending on the equipment, and operation instructions should be followed for the specific machine being employed.

Use a 4X objective lens and 300 µm thick flow cell. Some FlowCAM models transport the fluid using an internal syringe pump. However, this method may destroy meiofauna specimens; therefore, an external peristaltic pump is used for the flow path so that the specimens remain intact (Figure 2).

The image mode is set to Auto Image Mode. In this mode, all particles that pass through camera's angle of view are captured. The images to be captured in 1 sec is set to the maximum value (currently 20/sec).

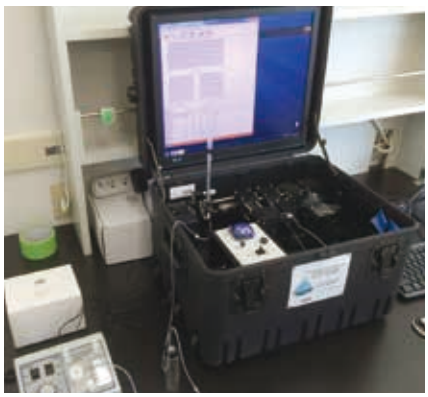

Figure 1 Picture of FlowCAM system

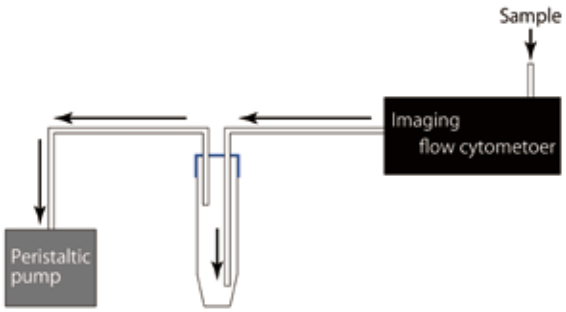

Figure 2 Example of flow line of imaging flow cytometer

1 Equipment and reagents

Imaging flow cytometer (e.g., FlowCAM)

50 µm size marker beads (Thermo Fisher Scientific Inc.)

Pasteur pipettes

Peristaltic pump

Ludox HS-40 (Ludox HS-40, Sigma-Aldrich, Inc.)

2 Procedure

1. Set the focus using 50 µm size marker beads. If the auto-focus feature is installed, follow the instructions supplied with the flow cytometer. If a flow cytometer is not equipped, set the focus manually.
2. Fill the flow line with Ludox HS-40.
3. Start the observation.
4. Introduce the sample using a Pasteur pipette while occasionally stirring the sample in the conical tube.
5. Conclude the observation when the entire sample has flowed through the flow line.
6. Rinse the flow line with water.
7. Transfer recaptured samples to a sieve, and use tap water to rinse the collected material into a new conical tube. Save and store as appropriate.

Notes Record information such as the magnification of the objective lens used and the created file name in a log (Table 1). Colloidal silica solution will still remain in the conical tubes with the recaptured samples. If colloidal silica solution is left to dry, it will form crystals centered on the meiofauna specimens as nuclei. Therefore, the solution should be immediately replaced with tap water and the samples saved and stored as appropriate.

A mixture with 2:3 ratio of colloidal silica solution and water can prevent drying and crystallization during the observation period.

Table 1 Example of examination log

Cruise ID: Date: Observer:

Sample name:

Water depth (m): Fraction:

Layer (cm): Fixation:

Log

Machine settings: Magnification:

Time	Description
	start
	stop

Image file name:

Memo:

chapter 4

Image analysis

4-1. Image analysis software

The analytical software VisualSpreadSheet is included with the FlowCAM system and is introduced as an example of image analysis software. This software creates an image library of any taxonomic group. It extracts images of similar shapes and colors based on the images of particles contained in the library, and has the ability to automatically classify matching images. This is effective when the morphological shapes are relatively simple, as is the case for phytoplankton, but is ineffective when the shapes are complex, as is the case for meiofauna. Even specimens of the same taxonomic group may pass through the flow cell at different angles, resulting in a wide assortment of varied captured images. Presently, classification by an experienced specialist is necessary, but as image databases for each taxonomic group are built and shared, people who are not as familiar with the organisms in question would be able to determine taxonomic groups.

Organisms were stained red (or pink) with rose Bengal, which allows us to pick the organisms using a sorting function by color (Figure 2).

1 Procedure

1. Start VisualSpreadSheet and open the acquired file.
2. Use the sort function to sort images according to the red/green ratio.
3. Go to the end of the sorted images and select images of organisms.
4. Save the selected images to a separate file.
5. Count the organisms by group at the taxonomic levels determined from the images (Table 2).

4-2. Other image analysis software

Images obtained by the FlowCAM are saved in TIFF format, which allows other image analysis software (for example, Zoolmage) to be used. In addition, methods to calculate meiofauna biomass based on image data are being developed (Baguley et al., 2004; Mazurkiewicz et al., 2016). By applying various image analyses and processing technologies, heretofore time-consuming meiofauna analysis can be accelerated.

At present, the sorting of images by an experienced specialist is time-consuming, but with the development of technologies such as Artificial Intelligence (AI), this process is likely to be accelerated.

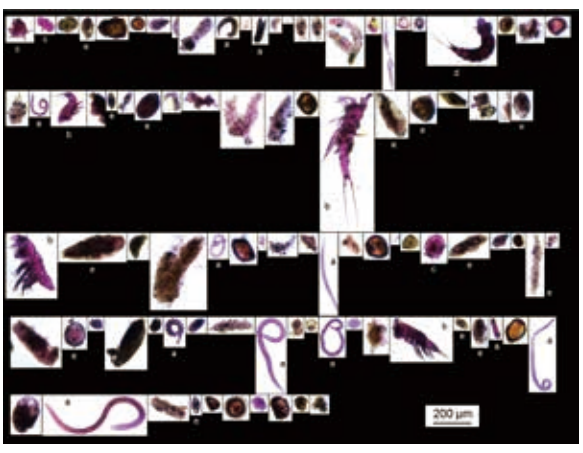

Figure 2
Meiofaunal images captured by FlowCAM. Labels in the figure indicate the meiofaunal taxa; a: nematoda, b: copepoda, c: nauplius larva, d: kinorhyncha, e: foraminifera. (From Kitahashi et al., submitted)

Table 2 Example of data sheet

Cruise ID: Date: Observer:

Sample name:

Water depth (m): Fraction:

	0-0.5 cm	0.5-1 cm	1-2 cm	2-3 cm	3-4 cm	4-5 cm	Total
Nematoda							
Copepoda							
Nauplii							
Polychaeta							
Bivalvia							
Ostracoda							
Kinorhyncha							
Turbellaria							
Oligochaeta							
Tardigrada							
Gastrotricha							
Cumacea							
Amphipoda							
Isopoda							
Tanaidacea							
Acarina							
Nemertina							
Others							
Total							

Memo:

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