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Synthesiology

English edition

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Effects of cooperation between a small and medium enterprise and AIST

Advanced ignition technology for the achievement of high thermal efficiency of internal combustion engine

Development of a rapid analytical system for glycans using a multistage tandem mass spectral database

Health care application of gas sensors

2015

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Highlights of the Papers in *Synthesiology* Volume 8 Issue 4 (Japanese version Nov. 2015)

Synthesiology is a journal that describes the objectives and social value of research activities that attempt to utilize the results in society, the specific scenarios and research procedures, and the process of synthesis and integration of elemental technologies. To allow the readers to see the value of the papers in a glance, the highlights of the papers characteristic to *Synthesiology* are extracted and presented by the Editorial Board.

***Synthesiology* Editorial Board**

Development of an in-solution observation method using atmospheric scanning electron microscopy (ASEM)
— Interdisciplinary research between semiconductor fabrication technology and biological electron microscopy —
The electron microscope is used in a wide range of science and technology fields, but it was difficult to conduct *in situ* observation of live cells because thin slices of samples had to be made and observed in vacuum. Ogura *et al* (AIST, Advanced Technology Division, JEOL Ltd.). created the development scenario for a device that enables correlative observation using both the optical microscope and the scanning electron microscope, and allows observation of living cells in solution under atmospheric pressure conditions. They succeeded by collaborating with an EM company and a research institutes that possess outstanding technique in manufacturing SiN films used in the sample dish window. Although this research was conducted with the goal of EM observation for living cells, one of the research results was the drastic reduction of conditions on the EM observation samples, and this opened the possibility for clinical application as diagnostic device for cancers and infectious diseases, as well as for observation of electrochemical reaction in solutions and reaction during temperature change.

Effects of cooperation between a small and medium enterprise and AIST
— Impacts of the idea of "Monozukuri" on technicians —
Komatsuseiki Kosakusho Co., Ltd., a medium-sized company, succeeded in the dramatic improvement of the micropiercing pressworking process through careful analysis utilizing AIST technology. Komatsu (Komatsuseiki) and Nakano (AIST) describe the case study from their individual vantage points, and discuss the factors that led to the successful joint research between a small and medium-sized company and a research institution.

Advanced ignition technology for the achievement of high thermal efficiency of internal combustion engine
— A demonstration of laser ignition in natural gas engines —
The gas engine that runs on natural gas fuel is considered promising for use in cogeneration. However, ignition by spark plug becomes difficult when lean combustion at high combustion ratio is realized in an attempt to achieve high efficiency. Takahashi *et al*. (AIST) show that the combustion condition can be expanded by pulse laser ignition to increase the heat efficiency of gas engine using methane.

Development of a rapid analytical system for glycans using a multistage tandem mass spectral database
— Toward an era where everyone can analyze glycan structure without specialist knowledge —
Glycans are molecules responsible for various biological functions in the body. Although they are being utilized in clinical diagnostic drugs, one issue is that it is difficult to analyze their structures. Kameyama *et al*. (AIST, Mitsui Information Industry, and Shimadzu Corporation) developed the technology for estimating the structure using the spectra database created from mass spectrometry of glycans, by adopting the respective technological specialties of the institutions or companies. The analytical system was commercialized according to the intellectual property strategy, and is now being used in several institutions.

Health care application of gas sensors
— Medical devices of breath analysis —
Shin *et al*. (AIST) developed the technology to detect and analyze odor components such as hydrogen and volatile organic compounds in the breath, using a low-cost, simplified sensor. Following the successful commercialization of the halitosis detector, they are engaging in the commercialization of other detectors with the cooperation of medical institutions and volunteer subjects.

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Contents

Development of an in-solution observation method using atmospheric scanning electron microscopy (ASEM)

— Interdisciplinary research between semiconductor fabrication technology and biological electron microscopy —

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Protein complexes in cells and tissues play critical roles in various physiological functions, including embryogenesis and signal processing. To observe the dynamics of protein complexes, high-resolution and high-throughput electron microscopy (EM) in aqueous solution is required. However, standard EM requires the sample to be in a vacuum. With ASEM, an inverted scanning electron microscope (SEM) observes the wet sample from beneath an open dish while an optical microscope (OM) observes it from above. The disposable dish with a silicon nitride film window can hold a few milliliters of culture medium, allowing various types of cells to be cultured in a stable environment. This system was used to develop *in situ* correlative OM/SEM immuno-microscopy in liquid. Using this method, we have observed a dynamic string-like gathering of STIM1 on the endoplasmic reticulum in Jurkat T cells in response to Ca^{2+} store depletion. We have also observed filamentous-actin (F-actin) and tubulin in the growth cones of primary-culture neurons, as well as in synapses. Further, we have monitored *in-situ* electrochemical reactions in electrolytes, and the melting and solidification of solder using ASEM.

Keywords : Correlative Light and Electron Microscopy (CLEM), microtubule, STIM1, immuno-electron microscopy, electrochemistry

1 Introduction

Many physical phenomena and chemical reactions occur in an aqueous environment, and our most distant ancestor was born in the sea. Therefore, to observe events in an aqueous environment at high resolution is important for both physics and biological research. For example, proteins are essential factors that support the biological function of our bodies, and many move dynamically inside cells. TRPV2 ion channels are normally integrated in the intracellular membrane, and when they sense a stimulus, they move to the cell surface and start to function within a few seconds.[1] In recent years, such quick acting proteins have been found in large numbers, and they perform physiological functions by associating with and dissociating from other proteins. Further, many protein complexes associate directly or indirectly with the cytoskeleton and are specifically localized in cells. Quick sample preparation and high-resolution observation are required to clarify the behavior and function of complexes with multiple components. So-called high-throughput EM observation would be such a method. However, in conventional electron microscopy (EM), complicated pretreatment procedures are necessary to prepare the sample for observation in the vacuum of an electron microscope.

The atmospheric scanning electron microscope (ASEM) that we developed is capable of observing cells labeled with heavy metal or fluorescence directly in water without pretreatment (Fig. 1). This allows high-throughput and high-resolution observation. It is also excellent for immuno-EM where the target molecule is labeled with gold-labeled antibodies, because antibody antigenicity is conserved in under aqueous conditions, and because the natural structure in hydrophilic conditions can be observed; hydrophobic treatment is not required. Moreover, ASEM can be used for correlative light and electron microscopy (CLEM), as it allows correlative observation of the sample by an optical microscope (OM) and by EM. The speed and high resolution with which it is possible to directly observe samples immersed in solution, are expected to enable various medical diagnoses. Although ASEM was developed for bio-research, the demands for observation in gas and liquid environments are high in the physical sciences and nanotechnology, and its applications are expanding widely.

2 Outline of conventional EM for gas and liquid and the ASEM system

2.1 Conventional EM and its limits

Epon thin-sectioning transmission electron microscopy (TEM) is used to observe intracellular structures and cellular organelles and molecular complexes, providing much higher resolution than conventional OM. This comparatively high resolution has allowed the discovery of various biologically

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and medically important mechanisms and phenomena. However, it is necessary to maintain the microscope column under vacuum to prevent the electron beam from being scattered. Therefore, the samples are normally subjected to pretreatments that may require several hours to several days to ensure durability in a vacuum. Since the treatments often involve dehydration and resin embedding, they could affect delicate hydrophilic protein structures and antigenicity. The development of an electron microscope that allows the observation of samples in a non-vacuum environment started from the demand for direct observation of samples in liquid or gas at high resolution by EM.

2.2 Environmental SEM

In 1979, Danilatos *et al*. developed the environmental scanning electron microscope (ESEM), which allowed the observation of samples in chambers filled with air maintained at low-density by combining a differential pumping mechanism utilizing apertures for the electron beam path and a low-vacuum secondary electron detector.[2] Since the observation took place in thin air at about 1/100th of atmospheric pressure, moisture evaporated from the sample during observation. Thus, the sample had to be imaged before it dried (Fig. 2 left). To resolve this issue, the sample was generally kept at low temperature, about 5 ºC, to reduce the evaporation speed of water.

2.3 The environmental capsule and its limits

The environmental capsule was born from the desire to realize a stable aqueous environment under high atmospheric pressure. It has a window that is transparent to an electron beam, and was developed for TEM and scanning electron microscopy (SEM). In fact, the first capsule was developed immediately after the invention of EM.[3] Carbon and collodion films were used for the early capsule windows. However, these environmental capsules did not become very popular due to the weakness of the films. When the film broke because of the uneven flatness of an area, the sample tended to contaminate the EM column. Recently, these films were replaced by strong silicon nitride (SiN) films or other films that were originally developed for the microfabrication of semiconductors,[4] and the environmental capsule evolved dramatically due to this increased stability.[5] However, the capsule interior is basically a closed system or a quasi-closed system with a volume of a few tens of microliters or less (Fig. 2 left). Sensitive primary cell culture directly from organs and the culture of nerve cells with high oxygen demand can be problematic, and neither long-term culture nor the addition of reagents from the outside are easy. Since the environmental capsule used for SEM only has one window, on the upper side, it is simpler than the capsule used for TEM, which has windows on both the upper and lower sides. However, cells and tissues tend to sink to the bottom due to gravity. Moreover, attaching tissue cross-sections to the film, labeling with antibodies in immuno-EM, and washing are not easy within this small container.

3 Development of ASEM, an inverted SEM

To overcome the above issues, we developed the ASEM (Fig. 1).[6] A strong 100 nm thick SiN film window (0.25 mm × 0.25 mm) was inserted into the bottom of a dish, and samples on the film were observed from below using an inverted scanning EM. Since the nomenclature ASEM has appeared several times in the history of EM development, we add some explanation. Initially, Danilatos referred to the

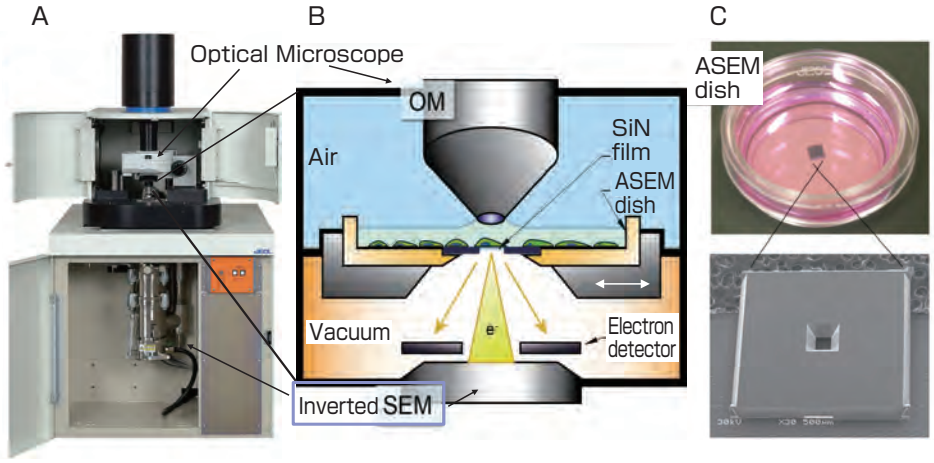

Fig. 1 Principle of ASEM

(A) The OM is installed opposite to inverted SEM, and the ASEM dish with electron–transparent film in the bottom is set between the two microscopes.

(B) The ASEM dish can be removed and used for cell culture in an incubator. The approximately 700 atom-thick SiN film (100 nm thick) of the dish was born from the semiconductor manufacturing process and is sufficiently strong to support a vacuum. The electron beam penetrates the film and irradiates the cells in the solution. The reflected electrons pass back through the film and are detected by the discoid BEI detector.

(C) ASEM dish. A square silicon chip is embedded in the center of the bottom. The lower image is the enlarged image of the chip as seen from the bottom of the dish. The center of the dish is etched to expose the SiN film window. The images are modified and reproduced from [11].

environmental (low vacuum) SEM as an atmospheric SEM (ASEM),[2] but the name of this device was later changed to ESEM. Next, Green and Kino[7] called the microscope in which a film is attached to the underside of a regular SEM column, where the electron beam is released, an ASEM, and Ackerley *et al.*[5] called the method that uses a regular SEM and an environmental capsule to separate sample and vacuum, ASEM. Our microscope is characterized by an inverted SEM that allows the observation of samples in a container that is open to the atmosphere, rather than within a capsule. Therefore, we decided to use the name ASEM for the fourth time because we believe it is an appropriate description for our atmospheric pressure EM and hope that people will use this name. Our ASEM is totally different from the Danilatos' ESEM, but closer to the environmental capsule in the point that a film is used to separate the sample from the microscope vacuum. Samples are observed in air or in water, at atmospheric pressure.

The 35 mm-diameter, removable ASEM dish, which is the sample holder we devised for our ASEM, enabled cell culture within a CO_2 incubator to mimic the gas configuration and humidity in our body. Moreover, due to this dish format, OM techniques that had already been developed, such as antibody-labeling and washing of cultured cells, could be utilized. Since in-solution observation was now possible, the time required to prepare samples became almost the same as for OM. The actual observation procedures are as follows. The cells are inoculated onto the ASEM dish and cultured in the incubator. After culturing for several hours to several days, the dish is placed onto the ASEM sample stage using the O-ring, and the interior of the inverted SEM column beneath the dish is vacuum pumped for about one minute. First, the behavior of the cultured cells is observed using the OM installed above the sample; this allows a wide field of view to be scanned at low magnification. Chemical fixation is done at the moment when desired cellular changes occur in the target cells, and the cells are immersed in 10 mg/ml glucose solution and observed at high magnification using the electron beam scanned through the SiN film from below (Figs. 1A, B). Since the component proteins are identified by labeling them with different fluorescent colors to analyze their distribution using the above OM, FluoroNanogold[8] or quantum dots[9] that have fluorescence and electron dense body can be used to label the antigens for correlative optical-electron observation.

3.1 Components of the ASEM

Figure 2 shows the scenario of ASEM development. The technological elements that comprise the ASEM are the electron-transparent film, the dish, the SEM and the OM. SiN film was adopted because of its strength. We optimized the semiconductor manufacturing process, and with the cooperation of Dr. Yoshiyuki Watabe of the Yamagata Research Institute of Technology and others, we succeeded in fabricating a SiN film window with a thickness of only 15 nm (about 100 atoms of Si or N). A CVD process and wet etching were employed to boost the fabrication precision, and we checked that the film would not break under pressure differences of up to 2 atmospheres.[6] The OM and SEM were designed to be positioned above and below this window (Fig. 2). The film has an open structure and was embedded in the bottom of a dish so that nerve cells with high oxygen demand could be cultured. It was important that the dish could be removed for cell culture in an incubator, and that high-throughput observation could be achieved by the use of

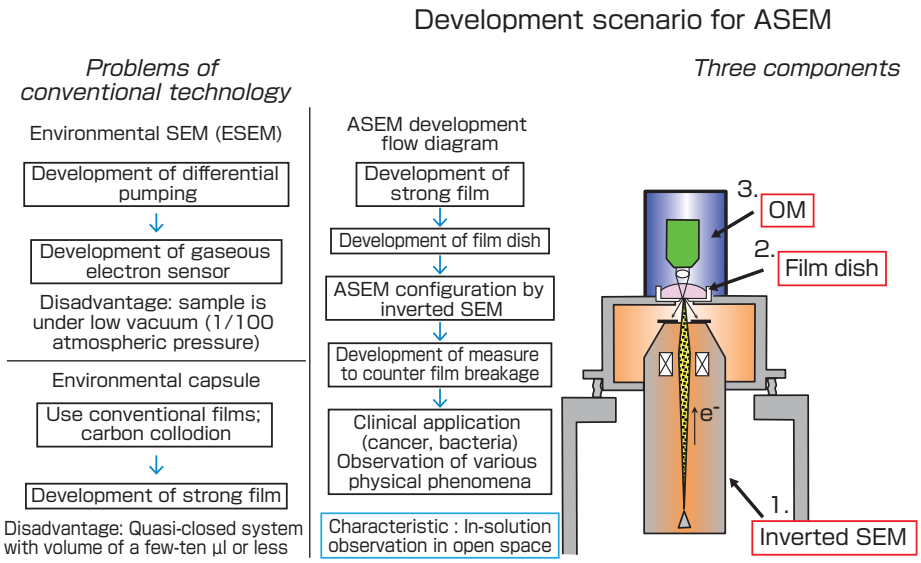

Fig. 2 Scenario for the development of ASEM

The goal is to observe samples in solution in an open space using SEM, and to enable application to clinical diagnosis and the study of physical phenomena. The basic development of the device involved the following: (1) to develop an inverted SEM; (2) to develop a disposable dish-shaped sample holder with a film window sealed above the inverted SEM; and (3) to enable simultaneous observation by OM and SEM by placing an OM above the dish. This figure was modified and reproduced from [6].

disposable plastic materials.[10]

3.2 Development of the ASEM (film) dish: Fabrication of the electron beam transparent film, its durability, and resolution

Part of the electron beam is scattered by the film. Therefore, the qualities of the film, particularly its thickness and evenness, are given as the factors that determine the resolution of the ASEM. As can be expected from the theory of electron beam scattering, the resolution gradually increased as the film becomes thinner. This tendency is clear at low acceleration voltages, where the penetration ability of the electron beam is low. However, in a commercial device, a 100 nm thickness is employed to reduce the possibility of film breakage and maintain a resolution of 8 nm. Moreover, using semiconductor manufacturing technology, we succeeded in developing an eight-window chip, and developed a dish with high observation efficiency by incorporating this chip into the device.

3.3 Mechanism of the ASEM

The SiN film seals the end of the inverted SEM column, separating it from the atmosphere and allowing the microscope column to be kept under vacuum.[6] The film is embedded in the bottom of the dish at the center, and the samples are either cultured directly on it prior to observation, or placed on it at the time of observation. The dish is doubly sealed to the stage by an O-ring at the side and a discoid rubber sheet at the bottom, and the evacuation is completed within about one minute. The electron beam irradiates the sample by penetrating the film from below, and part of the beam bounces back as backscattered electrons. The disc-shaped backscattered electron detector is placed at the exit of the SEM column. The backscattered electrons pass through the film, are quantified at each scan position by this detector, and are converted to images (Fig. 1B). The OM is installed above the sample, and same-field simultaneous observation is carried out using both microscopes. The main feature is that objects immersed in a large amount of solution (2 ml) can be observed. A defense system is present in case the SiN film breaks. Although the SiN film is strong, it can be accidentally pierced by sharp chips or other things.

3.4 Measures against film breakage

If the film breaks, a three-step defense mechanisms come into play as described below.[10] When the sensor beneath the film senses loss of vacuum, the shutter below immediately closes and the container above the shutter captures the liquid. At the same time, the air-leak valve functions, and stops further flow through the pinhole by setting the underside of the film to atmospheric pressure. Moreover, the liquid that passes before the shutter closes stops at the fine orifice through which the electron beam passes, located in the middle of the inner pipe of the column, preventing contamination of the electron gun. Because of these mechanisms, the ASEM can resume operation as soon as some parts have been exchanged. The interior of the column does not have to be cleaned.[10]

4 Application of the ASEM to cell biology

The cell cultured on the ASEM dish can be manipulated from the outside while it is being monitored by OM, and can be observed by SEM after fixation and staining. The resolution of the ASEM is 8 nm. The following experiments were primarily conducted to observe the organelles, cytoskeletons and molecular complexes of cells.[11] Immuno-labeling was achieved and applied to primary cultures of neurons; primary neuron culture is known to be difficult in a conventional environmental capsule.

4.1 ASEM observation of the cytoskeletons

The cytoskeleton determines the shape of the cell, acts as a scaffold for molecular localization, and provides rails to transport substances within the cell. The microtubules are formed by tubulin polymerization, and play important roles in cell division, flagellar movement, and the formation of neural circuits. Figure 3 shows ASEM images of microtubules of renal fibroblast cell line COS7 that were treated by chemical fixation, permeabilization using Triton™ X-100, primary labeling with anti-α-tubulin antibody,[11] secondary labeling with Alexa Fluor® 488 FluorNanogold™-Fab' antibody[8] and gold enhancement.[12] This series of procedures can be accomplished simply by changing the solution in the ASEM sample dish, and the task is completed within two or three hours. In the ASEM, the microtubules are mainly visualized

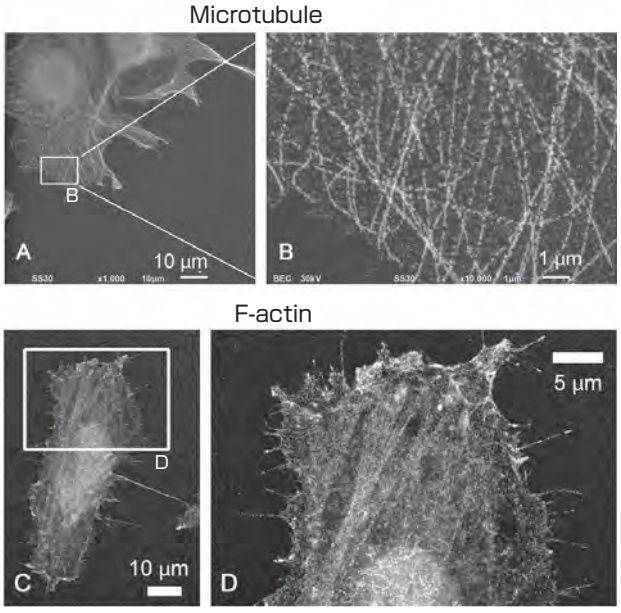

Fig. 3 Immuno-ASEM of microtubules and F-actin
(A) In-solution immuno-EM image where the α-tubulin of COS7 cell is labeled with fluorescence and gold (FluoroNanogold), and gold-enhanced. (B) At high magnification, each strand of the microtubule can be observed. (C) F-actin of Hela cell was labeled with phalloidin-FluoroNanogold, and gold-enhanced. (D) Enlarged image. Observation in low background without autofluorescence. The images were modified and reproduced from [11].

as white lines running from the center to the periphery of the cells (Fig. 3A). When enlarged, these lines are seen to consist of a series of gold particles about 20 nm in diameter (Fig. 3B). Note that the background is extremely low.

Of course, labeling for ASEM is not restricted to immuno-EM. Actin filaments (F-actin) are known to play an important role in the cell movement as well as in synapse formation and plasticity. Figures 3C and D show the distribution of F-actin in the cytoplasm and at the cell periphery. In this case, the F-actin of Hela cells was labeled with mushroom phalloidin-gold, and gold enhanced as in Fig. 3A. The actin bundles are thought to be mostly stress fibers.

4.2 Up to what thickness can the ASEM see?

When ASEM observation is conducted at an acceleration voltage of 30 kV, what depth from the film can be observed? As in Fig. 3, microtubules were labeled and comparative observations were made with a confocal fluorescence microscope. As seen in Fig. 4 A and B, the protrusion (arrow head) and the round structure (arrow) in the ASEM image were not present in the confocal image of the bottom part of the film (uppermost image). Rather, these structures were only observed when the sections sampled were at a distance from the film (the lower images of A). Together with other data,[13] this shows that a depth of 2-3 μm is observable by the ASEM.[11] The latter value decreased to about 1 μm as the accelerating voltage decreased to 10 kV.[13] Since synapses and neurites of cultured neurons are generally within this range, they are thought to be sufficiently observable by ASEM.

4.3 Observable depth predicted by electron pathway simulations

The trajectory of each electron was simulated according to scattering probabilities calculated by the Monte Carlo method. As the acceleration voltage increases from 20 kV to 30 kV, the irradiation depth of the electron beam increases (Fig. 5). Therefore, the observable depth is also expected to increase. The results agreed well with Fig. 4. In fact, the predicted observable depth at 10 kV is 1 μm, but deepens to 2~3 μm at 30 kV.[13]

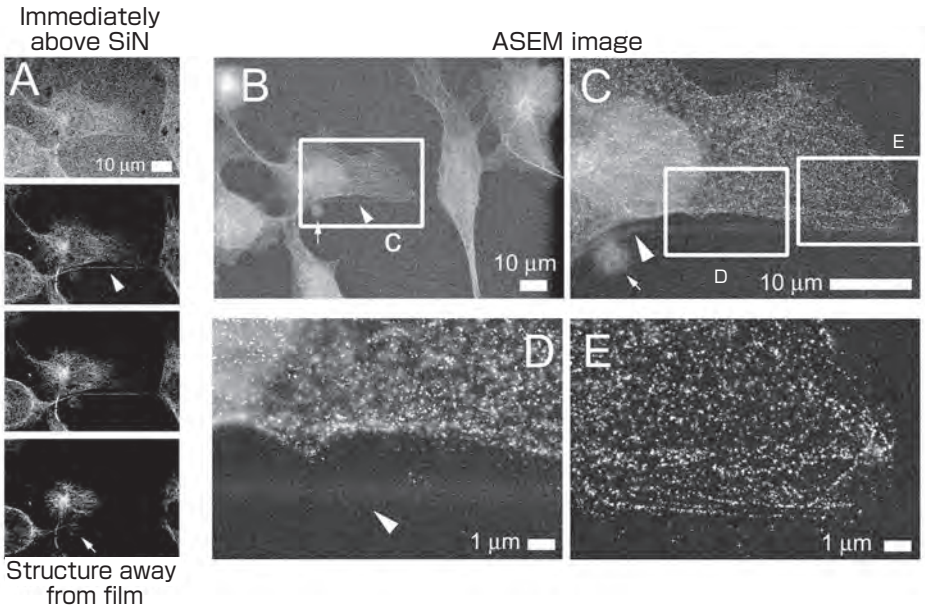

Fig. 4 Depth observable by ASEM

As in Fig. 3A, the α-tubulins of the COS7 cells were labeled with FluoroNanogold, and the depth observable by ASEM was measured by comparison with confocal fluorescence microscopy. (A) Confocal fluorescence microscopy image. Top image, the SiN film surface; bottom image, 1.32 μm above the film surface; central images, intermediate positions. (B-E) Corresponding ASEM images. It was estimated that 2-3 μm is the depth observable by ASEM.[11][13] The images were modified and reproduced from [11].

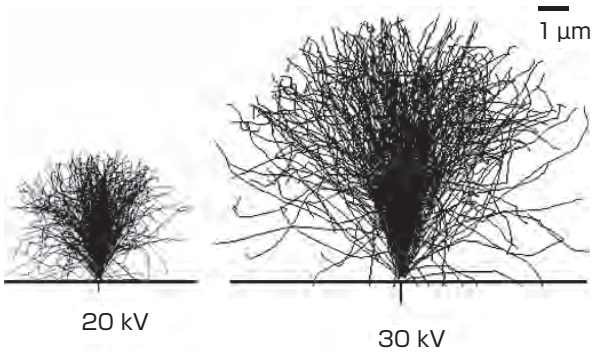

Fig. 5 Simulation of the electron pathways

The trajectories of electrons in bulk carbon were calculated by Monte Carlo simulations to understand electron imaging by ASEM. The simulations were made for acceleration voltages of 20 kV and 30 kV. The calculation supports the results shown in Fig. 4. The images were modified and reproduced from [6].

4.4 Reconstruction of the cytoskeleton during synapse formation in primary cultured nerve cells

The synapse is the basic unit of the neural network. Its size is small generally being 50-500 nm, and many of the axons and dendrites that comprise the network are minute. For their observation, the resolution of the EM is more appropriate than that of the OM. However, to count the synaptic connection of cultured cells by TEM, thin sectioning is normally required. This difficult job delivers thin horizontal slices cut against the culture plane after the neurons have been embedded in resin. On the other hand, ASEM allows observation of synapses without embedding or slicing, and we were even able to observe a small 50 nm spine.

Figures 6A-D show the observation of the growing axon terminal (growth cone) of a neuron. Primary cultures of the mouse hippocampal neurons (pyramidal neurons) were grown on an ASEM dish coated with poly-L-resin for four days,[11] and labeled for F-actin after fixation and cell membrane permeation. Then, the growth cone shown in the white frame of Fig. 6A was observed using the ASEM. Fine F-actin can be seen, developed like bicycle spokes in the lamellipodia of the axon terminal (Fig. 6C and D). Homer 1c coexists in the center of the spoke structure (Fig. 6B, yellow green). This agrees with the idea that Homer receives the Ca^{2+} signal information and intervenes with the action of the growth cone by controlling the actin polymerization. When the synapses were formed after 14 days of culture, the synaptic sites (Figs. 6G, H) could be identified using the Homer 1c (Fig. 6F, green) localized in the spine as landmarks. Microtubules existed as the backbone of the dendrites, and were hardly present in the synaptic sites (Fig. 6G). Also, it was newly discovered that microtubules inside the dendrite run diagonally (in a spiral) (Figs. 6I, J). It is known that the synapses are distributed spirally on dendrites to maximize space usage, and this could be the structural basis.

4.5 Dynamic rearrangement of signal transmitting molecules; visualization of the Ca^{2+} perception mechanism of the CRAC ion channel

The Ca^{2+} sensor STIM1, which is a membrane protein of the endoplasmic reticulum (ER), is the sensor of the plasma

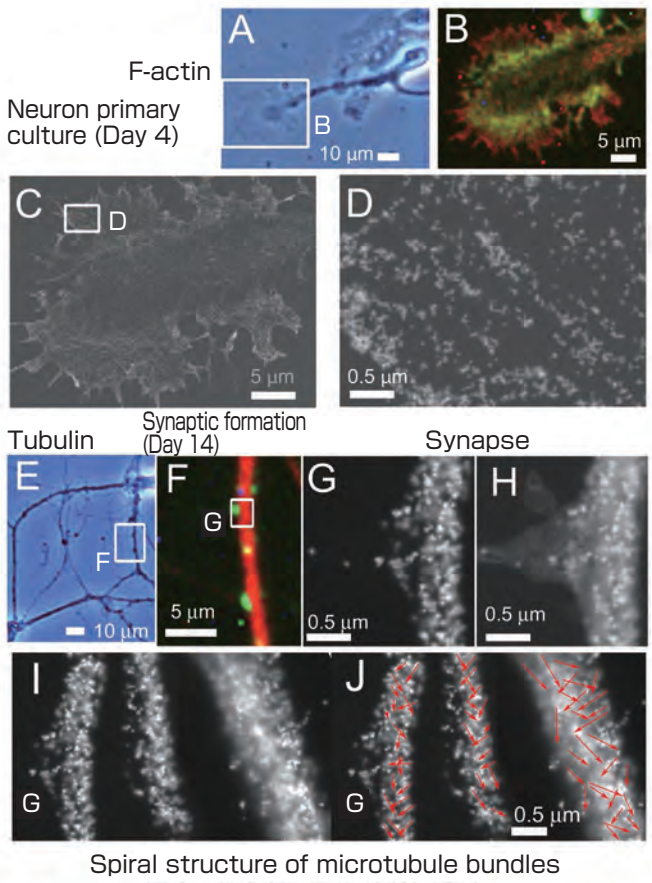

Fig. 6 Growth cone and synapse formation in primary culture nerve cells

Primary cultures of Homer 1c-EGFP transgenic mouse hippocampal pyramidal cells were grown on an ASEM dish coated with poly-L-lysine. (A) Phase contrast OM image of the growth cone four days later. The F-actin present was labeled with red and gold using FluoroNanogold. (B) Fluorescence images and (C, D) ASEM images after gold-enhancement. The F-actin was observed in a bicycle spoke-like form on the lamellipodia of the growth cone. (E) Phase contrast image 14 days after culture. Synapses had formed. (F) Fluorescent image. (G-I) ASEM images. (G)Tubulin of the synapse and (H) further stained with heavy metal. (I, J) The microtubules in the dendrites ran diagonally (in a spiral) against the long axis. Microscopy through a whole synapse and growth cone became possible at high resolution. The images were modified and reproduced from [11].

membrane CRAC ion channel. STIM1 monitors the Ca^{2+} concentration within the ER, and is thought to open the CRAC channel when it perceives the lack of Ca^{2+}. Figure 7 shows the observed distribution of STIM1 on the ER membrane before and after depleting Ca^{2+} in independent experiments.[11] In a steady state when the ER was storing Ca^{2+}, a correlative observation conducted by fluorescence labeling of the ER marker PDI (Fig. 7A) showed that the gold particles indicating STIM1 were distributed throughout the ER (Figs. 7B, C). Once Ca^{2+} was depleted, the STIM1 molecules gathered near the cell membrane in spots (Figs. 7E-G). The molecules gathered and joined together one-dimensionally making winding lines; the ASEM visualized this for the first time (Fig. 7G). Since the STIM1 molecule is asymmetric, the patterning suggests that the molecules bond head-to-tail. This STIM1 polymer bonds further with Orai of the cell membrane to form an active ion channel supercomplex. The positional changes of the CD44 glycan receptor on the plasma membrane, were also successfully imaged. This receptor is important in early development and cancer metastasis.[14]

5 Clinical application

5.1 Possibile use of the ASEM as a clinical diagnosis device

The use of the ASEM in clinics and hospitals as a diagnostic tool is expected, since long sample pre-treatment is not necessary and the direct in-solution observation of samples is possible. To visualize the characteristics that serve as the indices for diagnosis, we have developed heavy metal staining solutions for the following two cases, and have started to develop the diagnostic method. Further, to widen the observation area, we have also developed an eight-window sample dish.

5.1.1 Identification of infectious bacteria

Infection by bacteria is one of the most serious factors that threaten life. Since treatment differs according to the type of bacteria, a way of identifying the species that does not require time-consuming steps, such as culture, is strongly in demand. *Mycoplasma*, which is recently gaining attention as a cause of pneumonia and other diseases, has only about $1/25^{th}$ of the cell volume of *E. coli*. In the recent epidemic, over 90 % of the bacteria were antibiotic-resistant, and quick diagnosis is particularly in demand. However, due to the small size of *Mycoplasma*, diagnosis is difficult at all stages of disease, including early diagnosis. *Mycoplasma mobile* found in fish gills was observed with the ASEM as a model. After fixation and following membrane permeabilization, it was stained with heavy metal solutions consisting mainly of uranium and lead. The visualized bacteria had a cap structure at the more elongated end of the cell, nucleic acid in the round posterior end, and a varied structure in the middle (Fig. 8A).[15] These structures are characteristic, and are likely to become extremely prominent markers for diagnosis. When the "leg" gliding machinery that supports transport was labeled with gold-tagged antigens and gold-enhanced as in Fig. 3A, a waistband-like distribution was seen on the cell surface (Fig. 8B bottom), which indicated that the immuno-label may be useful for diagnosis. We also succeeded in the detailed observation of bacillus.[16] These results show that the ASEM may potentially speed up the diagnosis of infectious diseases.

5.1.2 Identification of metastasis; application to rapid intraoperative cancer diagnosis

During cancer resection surgery, intraoperative cancer diagnosis is sometimes required. The most important index for rapid cancer diagnosis is the size of the nucleus. Generally, tissues are frozen, cut into about 3 μm-thick

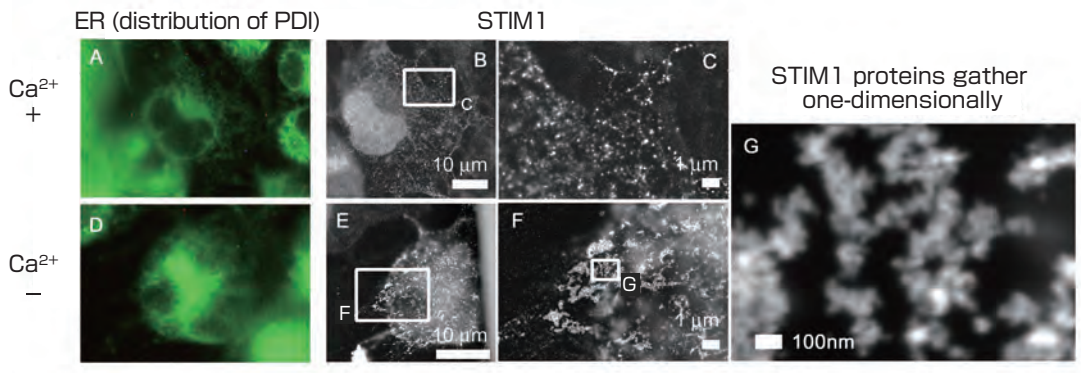

Fig. 7 Gathering of STIM1 proteins due to Ca^{2+} decrease in the endoplasmic reticulum (ER)

The STIM1 subunits that are the Ca^{2+} sensors of CRAC channels are distributed on the cell ER (A-C), and aggregate in spots at the puncta near the plasma membrane when they sense Ca^{2+} depletion (D-G). The colored panels are fluorescent images and the black-white panels are ASEM images of gold-labeled STIM1, and enlargements of the areas in white boxes are shown to the right. In the image with the maximum enlargement, the gold particles labeling STIM1 are joined together linearly, and we thus propose that the asymmetrical STIM1 molecules bond one-dimensionally (G). STIM1 is thought to form an ion channel supercomplex with Orai1 between the ER and the plasma membrane. (A, D) The ER marker PDI was fluorescent labeled. The distribution of the ER is shown. Here, we present the results of the COS7 over expression system, but similar string-like molecular gathering was observed for the intrinsic STIM1 in T cells,[11] and this phenomenon is considered to occur universally. The images were modified and reproduced from [11].

slices, stained with hematoxylin eosin, and observed using an OM. The extent of resection is sometimes determined from the nucleus size; cancer cells usually have bigger nuclei. However, the required cryo-sectioning is not easy, taking at least 15–30 min for all procedures per a sample. Since time is pressing during an operation, only a limited number of regions can be examined, which is also a problem. First, we developed, by trial and error, a staining method for the nucleus that can be used with ASEM. We found that the nucleus could be stained at high contrast when the cultured cell was fixed, first with 4 % paraformaldehyde (PFA) and then with 1 % glutaraldehyde (GA), and stained, first with 10-fold diluted Ti -Blue (platinum-Blue) solution and then with 2 % phosphotungstic acid (PTA) solution.[17] When the stained tissue was placed on the SiN film of an ASEM dish and imaged, nuclei 2-3 μm above the surface could be observed. We fixed and sectioned a normal lung and a breast-cancer metastasized lung from mice. The tissues were cross-sectioned into thick slabs, stained, and observed using the ASEM. The nuclei stood out as white structures, and the large nuclei of cancer cells were clearly discernable (Fig. 9).[17] Since this method does not require cryo-sectioning, it has the potential to dramatically speed up and simplify the intra-operative diagnosis. Observing as many areas as possible with the microscope is expected to increase the diagnostic accuracy.

6 Application to energy and material fields

6.1 Electrochemical reaction; application to battery development

Observing the electrochemical reaction in electrolytic solution *in situ* and at high resolution is important for the development of batteries and electrolytic solutions. Therefore,

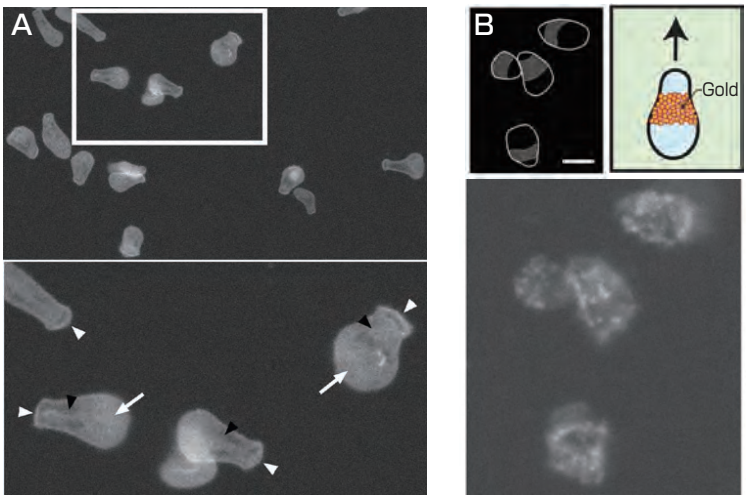

Fig. 8 ASEM images of *Mycoplasma mobile*
(A) Staining was done with heavy metal. The lower image is an enlargement. (B) Immuno-EM image of the foot protein complex Gli349. The cells were counter-stained with heavy metal after gold labeling. The upper image is a schematic diagram, and the cells move in the direction of the arrow. *Micoplasma* generally has 1/25th of the volume of *E. coli*. The fine interior structures of this cell were observed in solution for the first time using the ASEM. The images were modified and reproduced from [15].

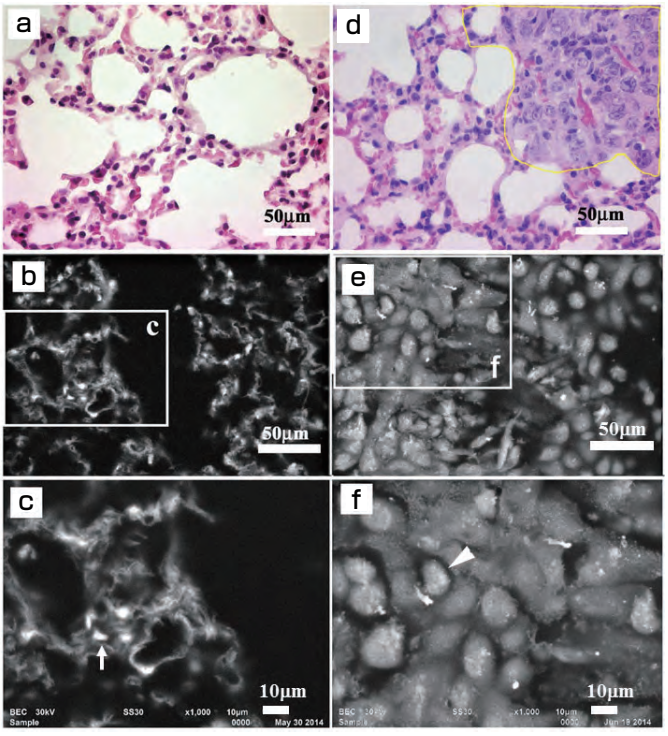

Fig. 9 Applicability of ASEM to cell nucleus-based cancer diagnosis of tissues
Comparative observation of normal lung and lung from mice metastasized by breast cancer. Nuclei near the tissue surface could be clearly observed in solution just by staining the tissue with Ti-Blue and phosphotungstic acid (PTA): cell cytoplasm was also weakly stained. (a) OM of an hematoxylin/eosin-stained thin-section of normal lung. Nuclei and the cytoplasm are stained blue and red, respectively. (b-c) ASEM image of independently prepared normal lung tissue slab stained with Ti-Blue and PTA. Alveoli with alveolar ducts, a vein system and trachea are visible. Normal size nuclei are observed (arrow). The tissue was placed on the SiN film and observed using ASEM. (d-f) Comparative observation of lung metastasized by breast cancer cells. Regular alveolar systems are only faintly discernable, but most of the space is occupied by cells of different shapes with larger nuclei (arrowhead), i.e., cancer cells. Because the nuclei near the surface of the tissue block could be observed, this method could be applied to intra-operative cancer diagnosis, which mainly depends on the size of nuclei. The images were modified and reproduced from [17].

we developed an electrochemical ASEM dish equipped with two electrodes set 100 µm apart on the SiN film (Figs. 10A, B).[18] The electrodes were made by sequentially depositing 10 nm thick titanium and 100 nm thick gold onto the SiN film by the sputtering method, and then processing the layers by photolithography and wet etching.

The area around the cathode was continuously observed in a saturated NaCl solution by ASEM under the following conditions: 5,000x magnification, 0.15 sec per frame, and four times integration. Immediately after monitoring, voltage was applied between the anode and cathode. The ASEM images recorded 3 sec (Fig. 10C) and 6 sec (Fig. 10D) after voltage application are shown. The growth of the tree-like structure from the cathode to the anode was recorded on video in real time. The sample was dehydrated after observation and the tree-like precipitates were analyzed by SEM energy dispersive spectrometry (SEM-EDS), and were found to be gold.

When the ASEM is used, the deposition of metal by electrochemical reaction in aqueous electrolyte can be observed in real time. The real time observation of electrochemical phenomena in electrolytic solutions in the vacuum of a conventional SEM is limited to solutions, such as some ionic liquids, that have a low vapor pressure. This is thought to be the first SEM observation made using a commonly employed aqueous electrolyte that is difficult to maintain in a vacuum.

6.2 Application to micro-wiring, melting and solidification of solder

To observe phenomenon that change depending on the temperature in solution and gas, we developed a temperature-controllable ASEM dish.[18] This has a similar structure to the standard dish, but includes a heater and a thermometer to change and control the temperature, and the body is made of titanium to withstand temperature rise (Fig. 11A). The SiN film could withstand the high temperature at which a type of solder (Sn: 42 wt%; Bi: 58 wt%) melted. At the temperatures shown in Fig. 11B, the solder goes from the solid to the molten state and *vice versa*, with accompanying morphological changes (Figs. 11C-D).[18] At a temperature of 145 ºC, the solder melted and the contrast of the ASEM image became uniform, and when it was cooled slowly to 130 ºC, the metals segregated (Fig. 11C). When the temperature was increased again to 150 ºC and then rapidly decreased to 115 ºC, the morphology of the segregation changed (Fig. 11D). The segregation varied depending on the cooling conditions. In electric circuit formation using solder, minute quantities of volatile ingredients such as pine resin are important, and the ASEM is expected to contribute to research and development in this field.

7 Future issues

The quick in-solution observation possible using the ASEM allows the realization of experiments under various conditions. Further, and of great importance, in-solution correlative observation by OM and EM can be accomplished by one device.

Since observation of samples open to the air and the use of

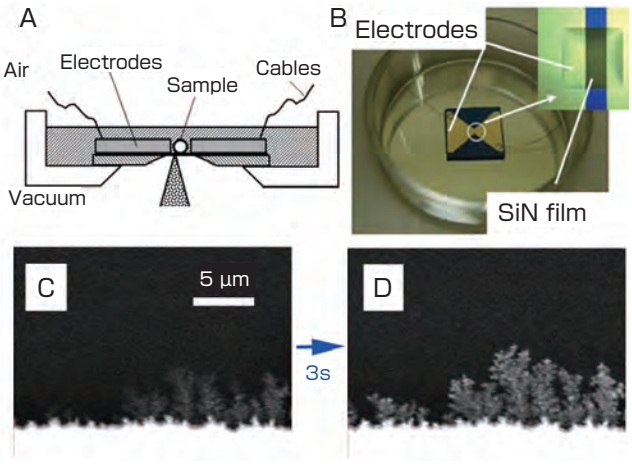

Fig. 10 Electrochemical ASEM dish (A, B) and the observation of an electrochemical reaction in the electrolytic solution (C, D)
Two electrodes were formed at a 100 µm interval on the SiN film (A, B). After applying 2.1 V, ASEM was used to observe the area around the cathode; after 3 sec (C) and 6 sec later (D). The dendritic precipitate grew from the cathode in the direction of the anode. The observation of phenomena occurring in aqueous electrolyte was shown to be possible. The images were modified and reproduced from [18].

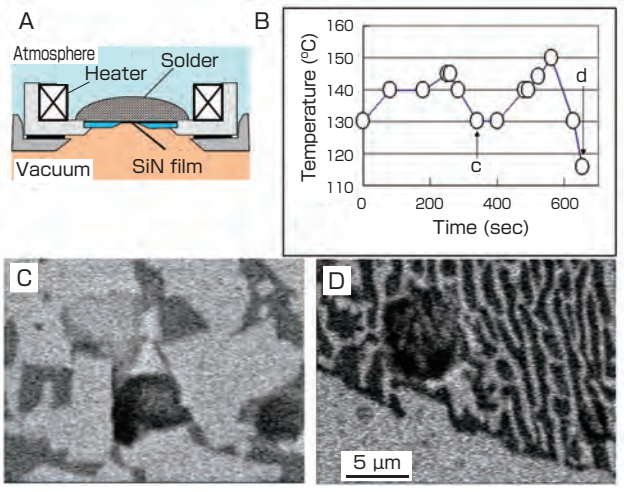

Fig. 11 Temperature changeable ASEM dish (A), temperature change (B), and ASEM images of solder (C, D)
The dish was equipped with a heater and a thermometer (A). The molten solder solidified as the temperature decreased (C, D). The bright-dark contrast stood out due to segregation, but the form differed according to the cooling conditions. Observation became possible even when volatile ingredients such as pine resin were present. The images were modified and reproduced from [18].

two microscopes was made possible by the development of the ASEM dish, it has become possible to observe various dynamic phenomena including the electrochemical reactions where gas is produced. For example, in an electrochemical reaction the bubbles produced float upward, so it is difficult to image them with the inverted SEM, but they can be observed by OM. At the same time, since observation is made from below using the inverted SEM, it is less likely to be affected by the bubbles, even in a phenomenon where gas is produced. Moreover, this microscope brings two new advantages to cell research. One is that various cells that were difficult to culture on an electron-transparent film in a capsule can now be cultured,[19] and the other is that the efficiency of labeling and washing is increased. In the ASEM dish, cells can be cultured in a CO_2 incubator in a relatively large culture volume (2 ml), compared to the small volume of the environmental capsule. Therefore, the effect of evaporation can be minimized, the ingredients of the culture solution can be maintained at a certain level, and the oxygen and CO_2 concentrations are stable due to gas exchange across a large liquid surface. Cell adhesion to the SiN film increases if the film is treated by a surface coating agent developed primarily for glass.[11] This is probably because the surface of the SiN film becomes SiOx during the manufacturing process, being oxidized by the fabrication process, and takes on a glass-like property. The ease of labeling and washing using the ASEM dish allows the immuno-EM method to have almost the same high-throughput as the OM. As a result, multiple-sample analysis and screening become simple, and there were cases where 40 dishes per day were observed. Since the preparation of the sample is conducted entirely in solution, the preservation of antigenicity is extremely good. We tested about 100 antibodies for cell labeling, and ASEM observation was possible with all of them. The majority of the antibodies used were mouse monoclonal antibodies, and it is expected that many commercially available antibodies can be employed. By adding the process of staining by heavy metal, it is possible to accentuate certain cell structures around the antigen. Uranyl acetate and PTA mainly accentuate protein and nucleic acid, while osmium tetroxide accentuates oil droplets and membrane structures.[6] By changing the acceleration voltage of the ASEM, it is possible to estimate the distance of the target to the SiN film. While this is similar to confocal OM in function, the principal is different.

High-throughput ASEM can observe diverse samples in a short time at high resolution. Compared to other EMs, the operation and sample preparation is extremely easy to learn. One factor is that, since the SiN film is set at the same height at all times, the focus position stays in the same place. As well as the results described here, the ASEM can observe fine protein crystals in a crystallizing solution without staining.[20] The application of ASEM to clinical diagnosis is one of our goals. It is expected that intra-operative diagnosis for cancer can be sped up by measuring the nucleus size with the ASEM. However, it is necessary to develop automated recognition and coloring software using image processing, since it is essential to distinguish between the nuclei and the cytoplasm, as in the standard OM-based diagnosis. In addition, ASEM is expected to contribute to the rapid diagnosis of infectious bacteria.[15]-[17] To accomplish this, observation protocols, including staining methods for general diagnosis mainly for pneumococcus, bacillus, MRSA, and viruses, are strongly in demand. The ASEM can be applied to drug discovery, food chemistry, polymer chemistry, cement, and other fields. Moreover, it can be applied widely to various fields of physics and physical-property research, such as materials science[18] and nanoscience.

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Discussions with Reviewers

1 Overall evaluation

Comment (Shingo Ichimura, Nagoya University and Hideto Taya, J-Space Inc.)

This paper discusses the construction of a scenario, the selection of elemental technologies, and the progress on the atmospheric scanning electron microscope (ASEM) developed by utilizing the ultrathin film technology for semiconductors, and its use in in-solution observation that is highly in demand in the bio field. I think this paper offers value for *Synthesiology*.

While the sample had to be kept in a vacuum in the conventional EM method, with the development of the in-solution observation by ASEM as described in this paper, the rapid and high resolution observation of the activities inside cells became possible for the bio fields. In the future, this method is expected to be applied to clinical medicine, as well as materials science and nanoscience fields.

2 Overall structure

Comment (Shingo Ichimura)

When I do a search on Google Scholar, the term and concept of atmospheric scanning electron microscope (ASEM) can be traced back to the 1980s. I think you should completely change the way this paper is written based on whether the main point of this paper is: (1) the development of ASEM, (2) the application of ASEM, or (3) both.

If it is (1), what is different from the conventional ASEM? What kind of new elemental technologies did you incorporate into the existing technology to compose the system (device)? What was the background that led you to this concept? If you describe these, I think the characteristics of the paper that deal with a synthesiological approach will become more apparent. Since you already have the description of the basic characteristic, please reorganize the paper while explaining the historical course of ASEM development, and clarify the characteristic of your development.

If it is (2), rather than listing the examples of observation, the paper will become a paper related to synthesiological approach if you explain the following: what were known using which method up to this moment (conversely, what were the limitations); what became known through the application of the ASEM method (in addition, what did you consider as the technological element to overcome the limitations of the conventional method); and how clear did your understanding of the essence of the observed subjects become.

Answer (Chikara Sato)

The main point of this paper is (3). Following your comment, I reorganized the paper to combine (1) and (2) as follows.

First, I totally reorganized and rewrote Chapters 1~3 considering the history of EM development. First, I added the development history of in-solution observation EM in Subchapters 2.1~2.3, and explained the limitations of the conventional method in Subchapter 2.1 "Conventional electron microscopy and its limit," Subchapter 2.2 "Environmental SEM," and Subchapter 2.3 "Progress of environmental capsule and its limit." I also added the origin of the name ASEM and the changes that occurred in the first paragraph of Chapter 3. For elemental technologies, I newly added Fig. 2 "Scenario for the development of ASEM" and some explanations in Subchapter 3.1 "Components of the ASEM." The developmental element based on how much the understanding of the sample progressed was also added.

3 Scenario

Comment (Shingo Ichimura)

The historical flow of the ASEM is now fairly clear. However, I think it is lacking in the point of "summarizing the outline of the scenario, selection of elements, and relationship and integration of the elements into one 'diagram of the scenario.'" Please add a single diagram that explains the elements (characteristics) and problems of the development methods, for which you have described the history (environmental SEM and environmental capsule), and present how the selection and deletion as well as the addition of new functions have led to this new development.

In accompaniment to the diagram, I think you should further explain how the characteristics "inversion of the SEM column" and "use of SiN film" were selected as elements that would be newly added.

Comment (Hideto Taya)

The scenario diagram shows the elements of the ASEM, and is insufficient as the scenario that should provide the framework of this paper. If the title of this paper is "Development of an in-solution observation method," you must show the course and process of research that led to the establishment of the in-solution method, and I think the development of ASEM was one of the steps. Please show the elemental technologies that were needed to attain the goal of the paper "establishment of the in-solution observation method," along with the issues that had to be solved.

Answer (Chikara Sato)

As you indicated, I reorganized Fig. 2. While showing the problems of the present technologies, I presented the flow of R&D to realize the "in-solution observation in open space."

4 Relationship between the title and "Introduction"

Comment (Shingo Ichimura)

The current title is "Development of an in-solution observation method." This title gives the reader expectations for contents about the clarification of reactions and phenomena in solution, as you show later in the examples of non-bio fields. On the other hand, the "Introduction" is clearly limited to the bio field, and does not state the significance or necessity of the "in-solution observation method." Therefore, it is necessary to reconsider whether to keep the title and change the "Introduction" (point out the significance and necessity of "in-solution observation method"), or keep the "Introduction" and change the title.

You do not need the chapter on "Discussion." This is a paper for a journal characterized by the discussion of synthetic approach throughout the paper.

Answer (Chikara Sato)

I worked on the beginning part of the "Introduction" and revised the whole paper.

5 Description that emphasizes the "application of ASEM"

Comment (Shingo Ichimura)

There are two non-bio field examples that you provide: one for electrochemical reaction and the other for micro-wiring. I don't think the latter, "melting and solidification of solder" is appropriate as an application example because the title is "Development of an in-solution observation method" (I think soldering is an in-gas reaction). In accordance to the title, please consider using (or deleting) this example or adding other examples.

Answer (Chikara Sato)

In circuit formation by solder, the minute volatile ingredients such as pine resin are important, and micro-wiring is a field where future development can be expected. Therefore, I used these examples as the possibilities of application. Upon reflecting on your indication, I added the expression, "In electric circuit formation using solder, minute quantities of volatile ingredients such as pine resin are important, and the ASEM is expected to contribute to research and development in this field."

Effects of cooperation between a small and medium enterprise and AIST

— Impacts of the idea of “Monozukuri” on technicians —

Takafumi KOMATSU[1] and Shizuka NAKANO[2*]

[Translation from *Synthesiology*, Vol.8, No.4, p.178-189 (2015)]

Case studies and problem-solving steps are more important than new-technology transfer when collaborative studies are conducted between a small and medium enterprise and AIST. Detecting causes and issues takes considerable time, but, experiences obtained during collaborative research can decrease barriers and speedup progress. This report presents a case study on a collaborative project between Komatsuseiki Kosakusho Co., Ltd. and AIST to find a way to increase the life span of micro piercing tools. The solution, indirectly obtained by overcoming another problem is cost effective and easily applicable to factories, though it was not the surface coating technique considered at the beginning of the study. Through such experiences, it is possible to continue collaborative R&D on this as well as other projects, e.g., within the “Operation Projects to Support the advancement of Strategic Core Technologies” framework of the Ministry of Economy, Trade and Industry.

Keywords : Cooperation, small/medium enterprises, problem-solving, manufacturing technology, joint research

1 Introduction

The major issue in conducting R&D for manufacturing technology at AIST is that AIST does not have a site of production. No matter what advanced technologies are developed, since there is no environment to implement and evaluate such technologies directly, they often end up as an “armchair theory.” Joint research with companies that engage in manufacturing is essential to prevent such theorizing and to execute practical R&D. Since there are many excellent companies still in Japan, it seems that a joint research can be initiated smoothly and the results obtained swiftly, but the actual situation is not so simple. Fujimoto states, “At many small to medium companies in the manufacturing industry in Japan, the three factors, ‘people,’ ‘things,’ and ‘money,’ have been so finely appropriated that the companies must engage in competition with very few options,”[1] and the resources of the companies have been depleted. In fact, in the age of corporate globalization process since the 1960s that includes (1) finding a footing in the local market, (2) exporting products abroad, (3) localized production, and (4) progression to multipolar production,[2] it was important for the regional small/medium enterprises to establish relationships with major core companies of that region to ensure their management stability. To sustain business in such an environment, the priority of the managers of most small/medium enterprises was to fulfill the demands of the large companies, and to respond appropriately to the large companies’ requests. The characteristic of small/medium enterprises was to focus efforts on specializing in certain fields as much as possible and to solidify their position within the hierarchy, and this was the most important concern of the managers.

However, the competition shifted to seeking the lowest possible cost of labor as a means to survive the cost competition. In the late 1980s, the IT industry that saw growth in the domestic market and enjoyed competitiveness faced the cost reduction race due to market saturation and low-price sales at mass retailers, and many companies opted to transfer their plants overseas. The barrier of borders became lower among the corporate managers. Moreover, small/medium enterprises themselves were forced to take the global market into consideration.

Due to communication revolution such as the Internet, it has become possible to send large amount of data such as drawings to remote areas. The efficiency of distribution stimulated the movement of people, and an environment developed where shopping could be done easily on the Internet. A new market was born. Now there is a method to publicize and advertise one’s skills, and direct business can be conducted with foreign customers by communicating in English that is not the mother tongue of either the customer or seller.

Recently, with the outflow of skilled workers through corporate transfer from advanced nations to emerging

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nations, and through the introduction of state-of-the-art facilities due to the decreased opportunity to hand down skills at domestic sites of production, the difference in technology between advanced and emerging nations is rapidly decreasing. Through increased communication speed of vast information, enhanced education in the emerging nations, and increased number of students coming to study in advanced nations, there is a possibility that the advanced nations will eventually be surpassed by the emerging nations.

Some of the regional small/medium businesses in Japan are starting to advance globally utilizing technologies that may be the foundation of other fields, with highly specialized skills and experiences that were gained at the site of production. To succeed in international competition and for the domestic manufacturing to survive, it is necessary to push up the technological advantage that is vanishing to the forefront and to regain the manufacturing technology that overwhelms others. Collaboration with research institutions is a way to adapt to the new environment, but collaboration is extremely difficult in reality.

In this paper, we report a case study of joint research between AIST and Komatsuseiki Kosakusho Co., Ltd. (hereinafter, will be called Komatsuseiki), a medium-scale company in Nagano Prefecture. The report includes the issues encountered at the site of production, how progress was made in linking the R&D without a site of production, what results were born including the changes in the "monozukuri (manufacturing)" mind, and what were the goals. Consideration is made on the effective ways of conducting joint research between research institutions and small/medium enterprises in the future. This paper is a merged work of Takafumi Komatsu, Executive Managing Director of Komatsuseiki and Shizuka Nakano, Advanced Manufacturing Research Institute, AIST. Initially, the plan was to divide the chapters and the authors write them individually, but we decided to merge the writings because each had different points for each topic. This chapter is also a combination of the manuscripts of the two authors. Therefore, please note that there may be places that may be difficult to understand.

2 History of Komatsuseiki (Taking independent steps from below a large tree)

Komatsuseiki was established in 1953 as an associate company for assembling wrist-watch parts for Daini Seikosha Co., Ltd. (currently, Seiko Epson Corporation). In its early days, it engaged in the assembly of watches as intended, and afterwards, retroacted upstream from manufacturing to production of dies to steadily widen its technological range.

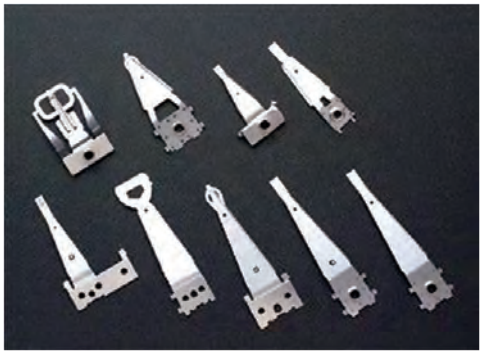
However, as the watch market became saturated in the latter half of 1970s and sales expansion could no longer be expected, Daini Seikosha recommended the company to become self-reliant on businesses other than wrist-watch parts. Fortunately, the company had fundamental technologies including pressworking, die polishing, cutting, and electric discharge machining for watch parts, and created a sales department necessary to apply such technologies to other fields. By responding to newly presented orders, the company was able to advance its pressworking technology for watches into the IT market.

The parts shown in Fig. 1, are the compact disk and hard disk suspension parts during the age when ten-megabyte hard disks were common. In addition to pressworking, a compound process was developed with laser welding that was state-of-the-art at the time done within the company to expand the technological range. However, the manufacturing time for dies could not catch up to the developing speed according to Moore's law, and with the overseas development of IT companies and the collapse of IT bubble in 2000, the IT parts business at Komatsuseiki declined rapidly.

Fortuitously, the manufacturing of automobile parts had started in the latter half of the 1980s due to the quality control technology that was nurtured in wrist-watchmaking. Since this involved supplying parts to products that directly affected human safety, the necessary quality assurance system was developed, as it was judged to be long-term, stable business. Also, since the issues of automobile parts were increased safety and environmental regulations, high-precision demands increased and the background was set for the utilization of wrist-watch technology.

The production scale of the orifices shown in Fig. 2, used as the electronic fuel injection parts for gasoline vehicles,

Suspension for HD (during pressworking)



Suspension for CD

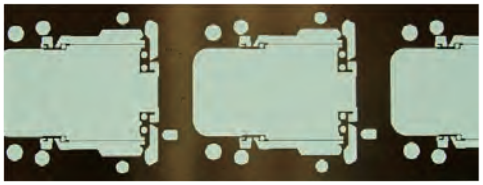


Fig. 1 Parts that were fabricated by Komatsuseiki by pressworking

increased steadily from the late 1980s, and increased to monthly production of 3 million units in 2000, and to monthly production of 5 million units in 2010.[3] In the course of such market expansion, with the strengthened environmental regulations that started from the Kyoto Protocol in December 11, 1997, the demand for small and high impact parts that were key to environmental measures intensified, and the advancement in technology and high-efficiency production were expected to be sought in the future.

3 Beginning of the collaboration

The beginning of the collaboration between Komatsuseiki and AIST was at the nano tech 2008 exhibition (International Nanotechnology Exhibition and Conference). An on-demand processing system[4] that was fabricated as one form of minimal fabrication technology was exhibited at the AIST booth (Fig. 3), and interest was raised there. The on-demand processing system was a small, automatic production facility that combined pressworking, aerosol deposition,[5] heat treatment, and others, and aimed for flexible production and manufacturing according to the product to be made. In the exhibition, the processing of a MEMS scanner was being demonstrated. For pressworking, there was also a poster exhibition on increasing the lifespan of the micro-piercing punch.[6][7] Extension of lifespan for the micro-piercing punch was an attempt to increase the lifespan 16 times by treating the surface with a unique technology of gold ion implantation. Komatsu, who was the Section Chief of Production Management at Komatsuseiki at the time, was expecting increased order of fuel injection nozzles, and at the same time, faced the issues of reducing the die maintenance time and increasing the surface area productivity to respond to the cost reduction demand.

From the side of Komatsuseiki, the explanation received at the exhibition seemed to provide a solution to the on-going issues. From the casual talks at the venue, a plan was born for a micro-piercing experiment, with the company providing a punch that was being created at the company, and gold ion implantation being done to the punch. From the decision that some direction could be gained from such "taste tests," this became the basis of the joint R&D.

4 Collaborative research and changes

4.1 Gold ion implantation punch experiment

We shall present a brief explanation on the punch lifespan extension by gold ion implantation. As an issue of the piercing punch, the process involves punching and piercing of the material and retraction of the tool. Therefore, the punch reaches its maximum compression load at the time of piercing, is immediately relieved of stress, and reverses after reaching 0 speed. At that moment, it will be subject to tensile stress due to the relationship with the material. Due to the halting of movement and load in the reverse direction, there are issues of frictional wear and adhesion to the contact surface. Moreover, since the stress on the tool is inversely proportional to the processing size, the stress is equivalent to the strength of tool material for micro-piercing. While coating is mainly done to reduce this load, AIST developed a surface modification technology using the ion implantation method. The ion implantation method is a technology for adding ions and elements inside the material, and it is able to change the surface condition as a continuous structure with no boundary layer. While the film forming technology for coating has the issues of exfoliation or size change, ion

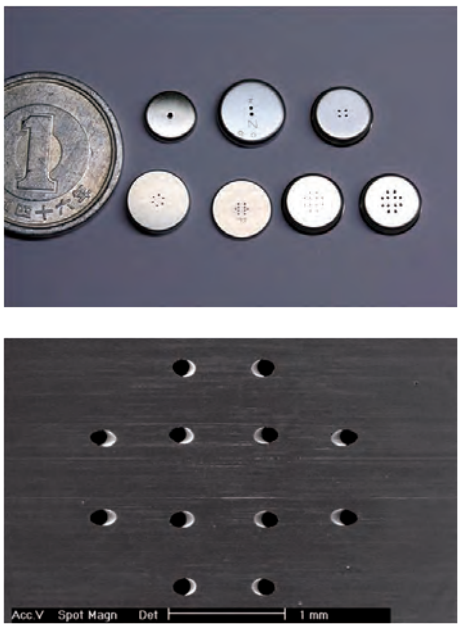


Fig. 2 Orifice plate for the gasoline fuel injection (top) and details of the orifice plate (bottom)

Fig. 3 Exhibition at the nano tech 2008
AIST booth in the Big Site venue.

implantation removes such issues.

To extend the lifespan of the die tools, we worked on the solution that it is effective to achieve "a condition that is stable throughout the repeated process in pressworking." Therefore, we sought possibilities to obtain continuity of the carbide sintering structure, to allow softening to spread the stress, and to reduce adhesion and wear. Gold has large mass number and can take larger collision effect during irradiation than tungsten that is a carbide material. As a result, a small amount of gold can destroy the crystals in large amounts, and it is capable of changing the carbide surface into a nearly homogenous amorphous condition at small irradiance of 1 x 10^{16} atoms/cm^2 and low energy of 75 keV. By conducting annealing treatment after implantation, the amorphous carbide surface changes into tungsten trioxide. Here gold ion acts as an oxidation catalyst and oxidization occurs to a deep level in a short time. The film of tungsten trioxide, which contains cobalt tungsten compounds and cobalt oxides in the binder material, has small Young's modulus, and the coefficient of friction remains stable for a long period. Moreover, there is the effect of reducing surface adhesion, and the results lead to the extended lifespan of the punch. However, to introduce this method to the site of production, it is necessary to evaluate whether it can withstand mass treatment in an actual processing condition. Moreover, there were issues of cost reduction and whether actual manufacturing was possible, and the evaluation tests were conducted through sample provision.

In the sample provision scheme, the product manufactured at Komatsuseiki could be used directly, and the gold ion implantation was done at AIST immediately. Since internal evaluation of the processing test using the punch at the site of production was possible, which enabled verification in a relatively short time, the collaborative tests were approved. As a result, however, the goal for extending the punch lifespan was not achieved in the orifice processing test for the gold ion implanted punch. As shown in Fig. 4, it is thought that the method did not improve the punch lifespan because piercing was done at a 30 degree angle using a punch of φ 0.2 mm, whereby the punch penetrated the material at an angle, the local stress occurred at the tip of punch, and the pressure spread out to the sides.

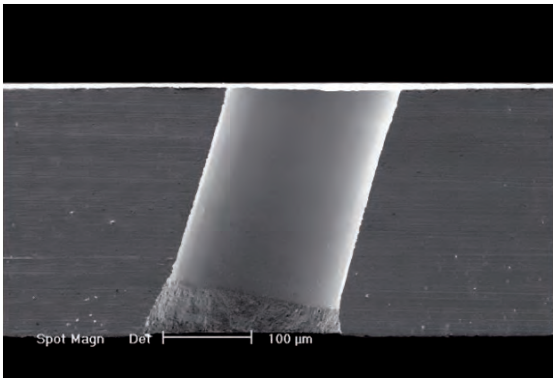


Fig. 4 Photograph of cross-section of angled piercing

However, by conducting this joint research, Komatsuseiki realized the necessity to break down the phenomenon that was considered as one process of "angled micro-piercing" into smaller processes, and to understand the individual processes. Therefore, to understand the phenomenon inside the die that was difficult to evaluate, such as the analysis of the cause of damage including punch wear and the processing capacity of φ 0.2 mm ultra-fine punch, the development of new evaluation methods was found to be necessary, and this led to joint research through a different perspective.

4.2 Punch surface observation and development of micro-piercing force measurement

While the visualization or bringing-into-sight of a phenomenon is the fastest method to find the cause of an issue and to seek the solutions, it is impossible to visually understand the phenomenon during die processing. In the past, studies have been done for experiments with glass, but sufficient evaluation could not be obtained since the conditions turned out different from the actual processing. Moreover, the evaluation at actual production volume would be even more difficult, and no study had been undertaken in the past. There was past research in which the die was disassembled regularly to remove the tool for evaluation, but in this method, the die had to be removed from the forming machine, disassembled, and then had to be reattached to the machine to continue the experiment. There was no reproducibility, it was difficult to conduct evaluation for every shot, and there was no case study for micro-piercing.

Therefore, AIST fabricated a die that enabled raising the punch (male part) to a visible position without taking the die apart, and fabricated an evaluation device that photographed the process with a microscope camera.[8]-[10] Figure 5 shows the outline of the device. In FY 2009, the project received the "Monozukuri" Subsidy for Product Development Small/Medium Businesses by the FY 2009 supplementary budget of the Ministry of Economy, Trade and Industry. Improvements

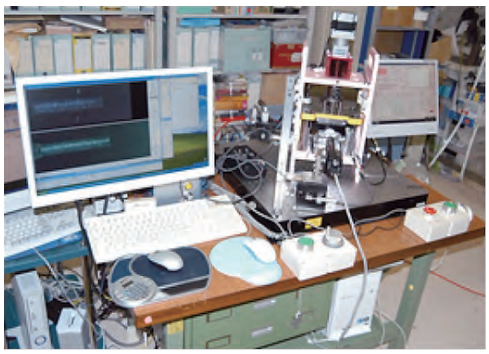


Fig. 5 Evaluation device for punch lifespan
Two cameras are used to observe the surface of the punch after every piercing shot.

were made to enable tests at speed surpassing 40 SPM (shots per minute). It then became possible to photograph all shots for the test surpassing 5,000~10,000 shots that was close to the actual production level. Also, a load cell was attached to the punch and a laser displacement meter to the forming machine, to enable monitoring the change in load-displacement. During the experiment, we were able to observe the phenomenon where the carbide punch underwent plastic deformation and then broke. Using this evaluation device, we set out to understand the phenomenon in a complex process of angled piercing in which the processing phenomena were unknown, and to clarify the causes for punch fracture.

In the joint research, we fabricated the experimental die for angled piercing that matched the actual processing at Komatsuseiki, conducted the punching test for 5,000~20,000 shots, and observed the process of change leading to punch damage. Since extremely high precision was required for die adjustment, the Komatsuseiki engineers visited AIST to jointly conduct the experiment while exchanging opinions. An example will be shown from the results obtained. Figure 6 shows the results of punch observation in the 5,000 shot piercing test and the load-stroke curve. The image shows the adhesion of worked material and friction damage in the punch tip. We were able to directly observe when and where in the punch the adhesion or wear occurred. Figure 7 shows the same experiment under the same condition as in Fig. 6, but scrap clogging, which is one of the main die troubles, occurred. The buckling distortion of the punch occurred (a), the fracture occurred at the base of the punch in the next shot (b), and observations and the load-stroke curve of the phenomenon are shown. In Fig. 6, the scrap push load was even and was 5 N or less. However, when the trouble occurred in Fig. 7(a), the scrap push load increased to 18.6 N. Although it is difficult to see in the image, slight warping occurred in the punch, and then it buckled. In Fig. 7(b), the scrap push load surpassed 20 N, and the load was rapidly lost in two steps. Then the load decreased vertically. The punch broke at this point. While not shown here, the situation leading to the destruction of the punch was obtained as data, and the process and the moment of punch fracture were clarified along with the cause. From the results obtained in these tests, we created the method for detecting scrap

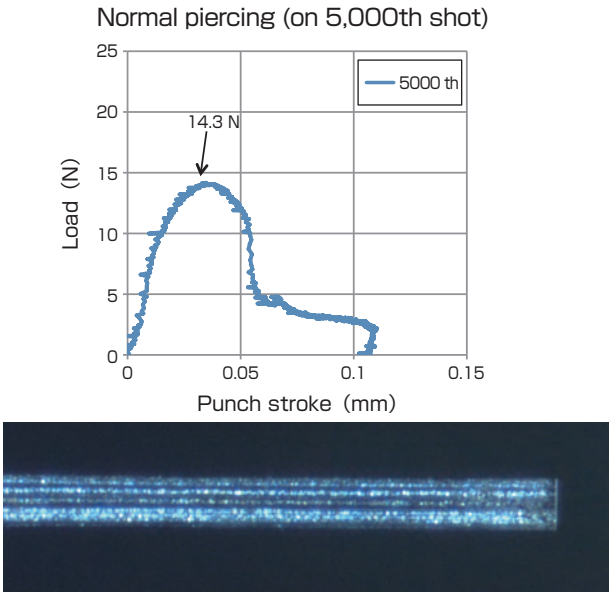


Fig. 6 Load-displacement curve during micro-piercing obtained by the evaluation device shown in Fig. 5, and the image of punch (the case when piercing has been done normally)

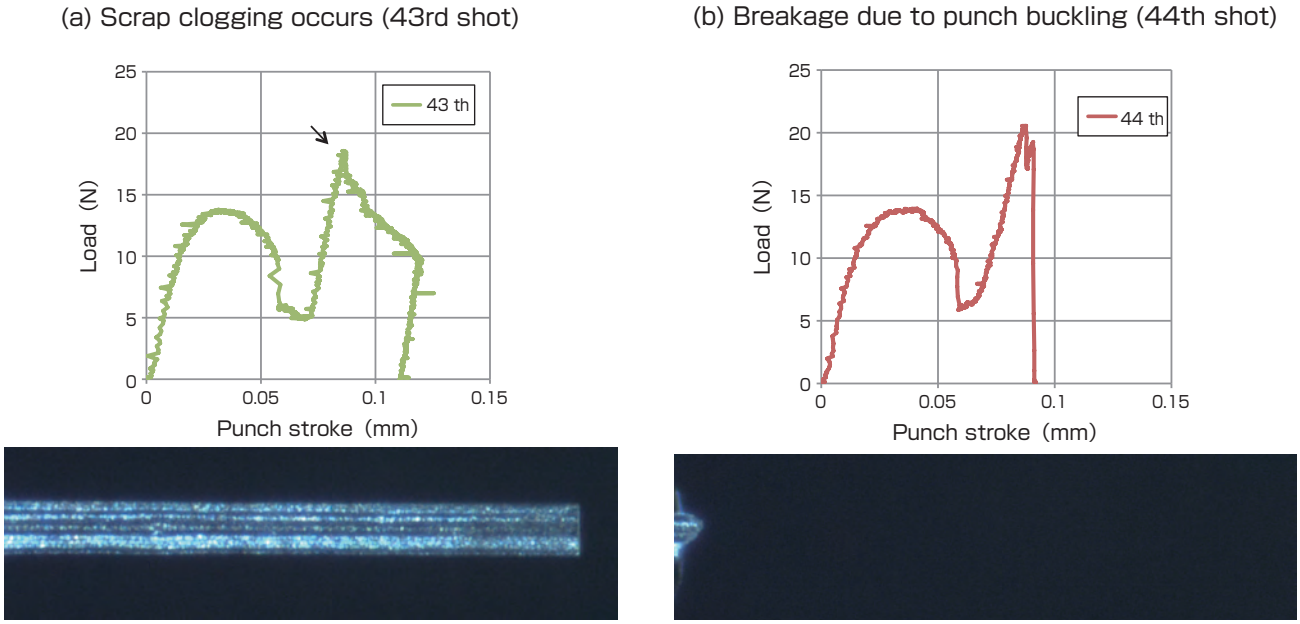


Fig. 7 (a) 43rd shot when the scrap pushing load (arrow) is high due to scrap clogging, and (b) 44th shot when breakage occurs at the base of punch due to buckling

clogging and established countermeasures.[11]

The experiences of visualizing and understanding such a phenomenon were unprecedented even in companies that have specialized in pressworking, and we shifted to funded joint research as the expectation rose for problem solving through further research. As a result of repeated experiments, it was found that the maintenance level of the die was reflected in the changes in punch surface, and it became possible to evaluate the level of clearance adjustment in one shot. Although the clearance volume was only a few microns, an integrated evaluation tool was obtained considering the precision error of punch and die, slight eccentricity, positional precision with the die, imposition error, and others. Clearance is small in microprocessing, and the error relation with the precision of die making is very tight. In such a situation, we recognized that the effect of positioning during exchange of punch and die is very high. To be able to easily determine the condition of the die before processing continues contributes highly in reducing the burden of the maintenance worker. Also, as shown in Fig. 8, by preparing the material in mid-process and evaluating the distortion and hardness of the cross-section, we were able to clarify the state of materials

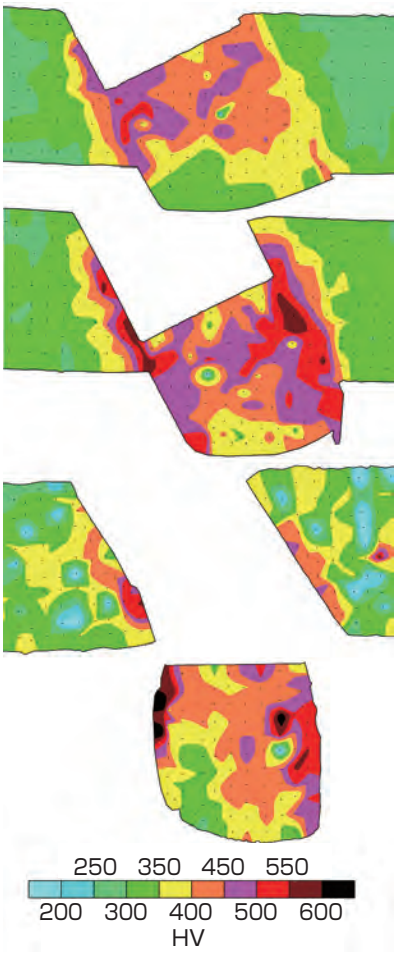


Fig. 8 Change in hardness distribution in the cross-section in angled punching[12]

that would become the product.[12] Such visualization of processing enables breaking down the phenomenon of piercing that was thought to be a single process into four processes: (1) punch entry, (2) shearing, (3) pushing in the scraps, and (4) punch retraction. It then became possible to separately analyze the issues for each process.

Other diverse problems and phenomena are being elucidated, and it is desirable that solutions are generated that fill in the gap between research and actuality, such as cost consciousness, practicality, and attainment of reproducibility and stability. For example, anything that accompanies change in the procedure must be approved by the downstream user, and any addition to the existing procedure may be difficult depending on the content. Therefore, a method that can attain effectiveness without changing the procedure and can be accomplished by reviewing the current work method is likely to be employed. In this joint research, many findings were obtained for the initial issue, and the solutions including reconsideration of die size and thorough management involved hardly any additional cost, could be done in the company facility, and enabled repeated restoration to the same condition.

In manufacturing, the essential goal is to obtain a product and that it is desirable that it be of high quality, stable, and of low cost. On the other hand, research provides the means for a solution, but the means and the end are often confused. For example, even if the extended lifespan by surface treatment is superior and should be promoted, to be used in corporate production, equipment must be introduced, treatment condition must be optimized, and evaluation must be conducted during production. Only after such steps, the production can be started, but often the time required for such introduction is not available. It is necessary to discern the goal that the company demands and to provide diverse means. The difficulty of R&D in manufacturing technology lies in this point, and while solutions cannot be obtained if one becomes involved in the method, research is not possible unless one becomes involved in the method. We believe it is effective to seek solutions while generating temporal gaps such as steadily going forward while taking diverse methods, extracting issues and problems in the company based on the accompanying analysis, and then taking the next step forward. While the results may be difficult to understand as tangible results may not be obtained or the result may not be clear at first glance, seen in the long term, it will be highly effective for the Japanese manufacturing industry.

This joint research progressed from the consideration of the processing method to the consideration of materials. Materials with fine metal crystal size were prepared, and the evaluation tests on how they would affect the process and product were started. Also, by analyzing the micro phenomena such as changes in the crystal structure during processing, the quality

and processability of products were improved, which then developed into the manufacturing technology of advanced products. This led to the new phase, and the company strengthened its analysis environment by introducing the electron back-scatter diffraction (EBSD), and strengthened support of manufacturing by the construction of a system that allowed provision of test data to fulfill the customer's expectations. From FY 2013, industrial support themes were selected, and the development of varied shape micro-piercing, which demands a more advanced piercing process, and the development of metal micro-pumps that utilize the diffusion bonding technology[13] were started. Here, based on the results obtained in the aforementioned joint study, we aim for high-quality products by developing a die that incorporates a nanometer precision positioning stage that enables assembling precision and ease of maintenance.[10][14]-[16]

5 Development in the corporate site of production

Figure 9 shows the cause-and-effect diagram of pressworking for precision dies before the collaboration. The affecting factors are basically the 4M, or man, material, method, and measurement, and each factor is followed individually in detail. The site of production was in charge of the part concerning people, the data provided by the material companies were used for materials, the die parts from the engineering division were incorporated, and the measurement device and production facilities selected by the production technology division were used to manage the quality control of the product.

These methods are set by the quality control standards such as ISO9001. No objections are likely to be raised as long as the company strictly follows and practices this ISO that has been translated into various languages. However, from a different viewpoint, since it is set by ISO, it is possible to be conducted by companies throughout the world, and it is not a factor that contributes to competitiveness.

By showing the flipbook animation of the punch surface and the change curve of the processing capacity during angled micro-piercing mentioned earlier, a certain reaction occurred at the site of production. By being able to visualize a phenomenon that could not been seen before or could not be explained before by words and images, the common expression of processing was now possible.

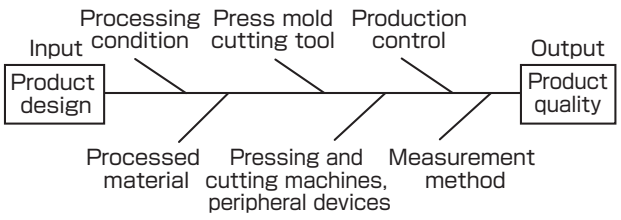


Fig. 9 Early cause-and-effect diagram

Through collaboration of joint research, a method different from trial-and-error that was done conventionally was developed, where the current situation was analyzed, the problem was defined, the solution was hypothesized, and the verification was done. When the test result was presented in the company, a certain change occurred not only among the researchers but also among the on-site workers. Until then, "angled micro-piercing" was considered to be a single process, but after the presentation, they started analyzing the process by separating it finely into (1) punch entry, (2) shearing, (3) pushing in the scraps, and (4) punch retraction.

Figure 10 shows the cause-and-effect diagram of the occurrence of burrs that are formed during angled micro-processing when processing orifices. This diagram was made and presented by the line manager. Compared to the earlier cause-and-effect diagram shown in Fig. 9, the problematic phenomena are refined and the depth of understanding and expression have changed dramatically. By refining the phenomena that were construed in only a few categories, the core cause is pursued and it has become possible to take specific actions for its solution.

The result greatly changed the management index called the "first run rate," that is the probability of a product becoming a completed product without becoming defective from the initial stage to the intermediate stage in a production lot. In 2011, the first run rate of Product A was less than 70 %. There were more than a dozen procedures, and 30 % of the product that were produced in the first run became defective in a procedure somewhere along the way.

To overcome this situation, we looked at the activity steps including the on-site activities. We looked at the manufacturing process and management method of the product, difference in measurement by people, all the way to the movement of the punch during processing. For example, measurement of the hole position was done manually using tool microscopes, but because there were individual differences, we worked to reduce personal differences by shifting to image measurement.

Such action goals were led by the production site personnel under the slogan "100 % One Path Success." After four months of action, 90 % was achieved stably, and 100 % was achieved in five months. After that, over 95 % is being achieved, and this site is positioned as a model line for training other workers and freshmen.

The results of joint research were shared through in-company presentations. In 2013, we succeeded in extending the punch lifespan by changing the size of part of the die and attaching the control standard. This was the initial goal when we started the collaboration for the research of the gold ion punch, but we reached the result by continuing the study

using alternative methods and analysis, even though the initial method did not yield the desired effect.

6 Changes in improvement thinking at the site of production through collaborative research

It is difficult to set R&D divisions in regional small/medium companies in terms of budget. A division that does not produce products is considered a division that does not earn money, and that brings about decrease of human, monetary, and material resources for research.

Komatsuseiki has 230 employees, and looking at the education level of the workers regardless of their ages, currently there are two people (0.9 %) working toward doctorates, five (2.1 %) with graduate school degrees, and 21 (9.1 %) who graduated from college. About 12 % of the workers in the company have been involved in research. Of course, many have gained on-site experience after joining the company, and have engaged in problem-solving research, but most has resulted from customer demand, and it is difficult to conduct R&D based on their own social or market projections. Even if there are researchers, there are only few manufacturing companies that actually engage in research, and how to fulfill customer demands is the priority for obtaining their daily bread. While the number of employees is increasing due to increased production scale and sales, the number of researchers are actually decreasing in companies that specialize in manufacturing.

To solve such a situation, it is important that the manager or the people close to management engage in collaborative research and share and develop the results in the company, in order for the small/medium manufacturers to expand to new areas. Particularly, when the scale of the company is small, the ability of the top managers tends to equal the ability of the company. Therefore, the overall level increase is accomplished efficiently in terms of cost through top-down rather than bottom-up, as the plans can be executed by short-term decisions. By creating success cases through small start-ups, more people can participate, the construction of collaborative body becomes easier, the consensus of company organization can be gained readily, and therefore, the scale can be increased gradually.

However, since the combinations of the talents of individual

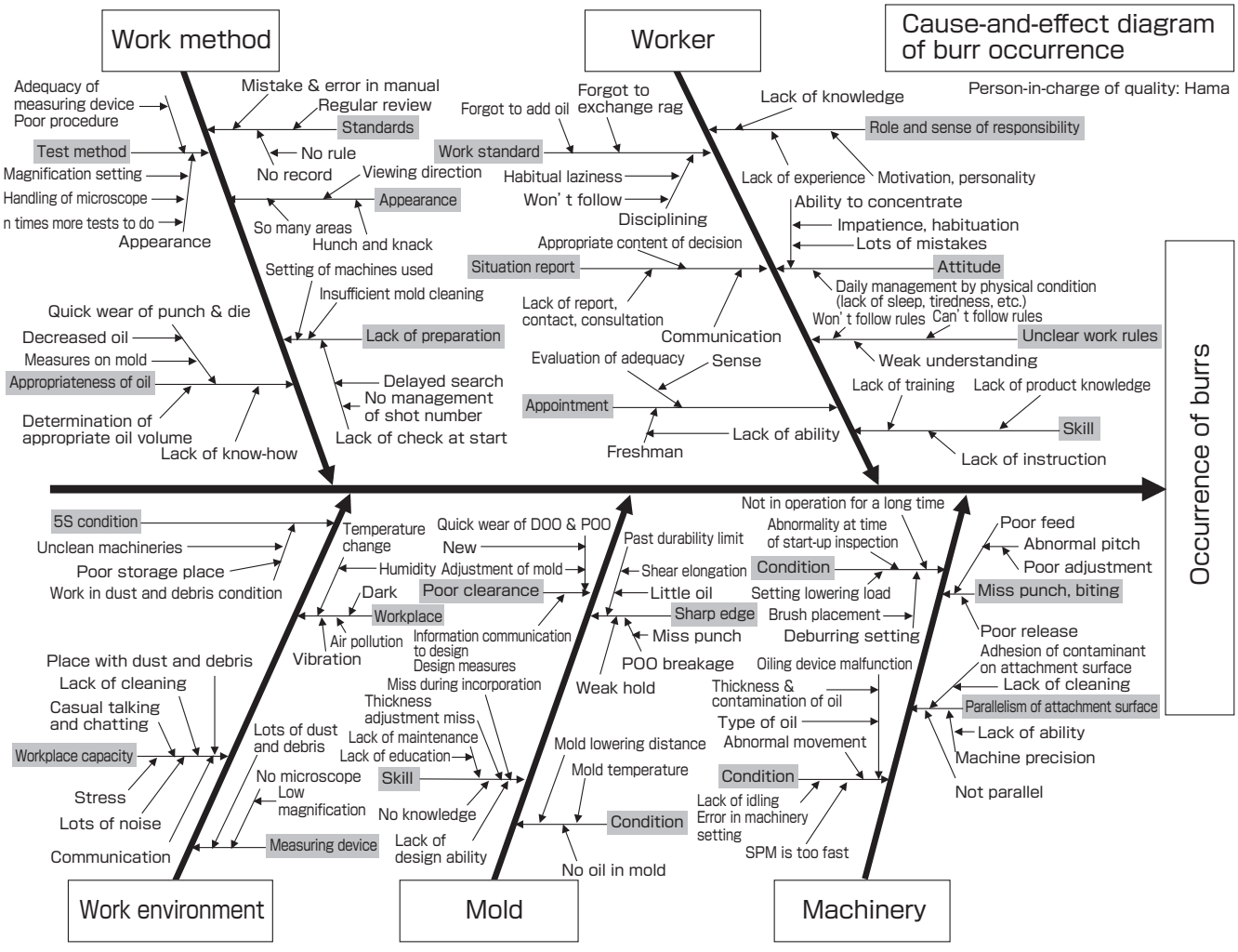


Fig. 10 Fig Detailed cause-and-effect diagram that focuses on the problem that occurred

collaborative members who understand the market demand is infinite, the burden on the person-in-charge will be very high. In a small/medium company, only the top manager will be able to withstand such burden.

In joint research between small/medium businesses and research institutions, in many cases, the research is narrowed due to the focusing of the issue. This tendency is strong particularly in the joint research with universities, and there is a tendency to set one solution per one issue. However, in the research with AIST, solutions are obtained through multiple, comprehensive methods that are studied concurrently. In a small/medium company, since one person must cover much ground, research that runs close to the site of production is necessary as in this case.

At Komatsuseiki, the understanding deepened that new customers can be gained through the development of research results, and the collaborative research activities expanded the company's activity range including procurement of competitive budget, patent strategy, and participation in other projects through the relationship with the collaborative partner. In June 2013, a new R&D office was opened, and the evolvement of research results is being done through the establishment of a new affiliated company.[17]

Seen from the side of AIST, there are plenty of research cases, but the application of results is difficult. Particularly in the development of manufacturing technology, it can be said that AIST does not have hands-on material because it does not have a site of production. Therefore, corporate joint research is important, but problems begin with terminology. For example, in research, "micron (micrometer)" is used regularly, while the company uses "x y-th" where the expression is in fractions based on millimeters. "1 μm" will be "one one-thousandth." Since the terminologies used at companies and research institutions are not necessarily the same, communication is difficult. In fact, as in the example of size explained above, the researcher construes the size "50 μm" as 50 times 1 μm, while the company considers it "one-hundredth of 5 mm" or perceives it as half of one tenth of 1 mm. Although the values are mathematically the same, the cognition is different. Since tolerance is utilized at the site of production, the "rough values" are also accepted as standard. In terms of awareness of numbers, for a researcher, 1 μm against 50 μm means that something is off by 1 μm or there is a 2 % difference, while on site, one one-thousandth against five one-hundredth seems to be a small difference. However, the production site has more experience struggling to process something at precision requirement of one one-thousandth scale. Nevertheless, in the joint research with Komatsuseiki, this gap in perception was significant. However, through monthly meetings to conduct joint experiments with the engineers, the communication level was mutually improved, and the researchers and engineers who could understand both perceptions were born. This is the greatest product of this joint research. In the technological discussions with Komatsuseiki, currently the discussion normally extends to the nanometer order, but people can switch freely from the expressions 100 nm or one one-ten-thousandth in their discussions now. In future research topics, new processing and manufacturing technologies are being developed. These will be works of near-boundary and boundary region surpassing the framework of current technology. In such cases, further communication ability will be required. From the age when one did not have to know Newtonian mechanics, one has entered the age when quantum mechanics is regularly used, and the agreement of perception, not only knowledge, is necessary. To accomplish this, it is necessary for people to engage in one experiment and be able to synchronize. Regardless of how excellent the results may be, transferring technology is not that simple.

7 Conclusion

The advanced processing technologies that were taken up by regional small/medium companies during the rapid economic growth period after World War II have become caught up in the globalization of companies that lead the game, the technological revolutions exemplified by the Internet, and the economic problems such as the Lehman shock. It is mandatory that they adapt to such environmental changes. The regional small/medium companies are exposed to global competition without exception.[18] To maintain the competitive edge in such an environment, problem solving by collaboration rather by individuals is one method for competitiveness and differentiation. In companies that have little change in employee composition, there is a limit to the problem-solving abilities and it is difficult to shorten the distance between the customers.

To capture the diverse or niche demand of the customers, it is necessary to deepen understanding of the people with whom one might collaborate and their skills and knowledge, and to work together organically. This will enable building of competitiveness unseen before, and shall also create new customers.

Many regional small/medium companies are reluctant to promote collaborative research due to the limit of "people," "material," and "money." However, by building the research environment through small start-ups and then expanding and developing into the site of production, it is possible to obtain effectiveness in various new ranges.

From the side of research institutions such as AIST, collaboration with small/medium companies that engage in manufacturing is essential. On the other hand, research institutions tend to think that the results can be used as is, but many of them are indirectly related to manufacturing.

At actual sites of production, manufacturing methods are different for each product actually made, the issues are of infinite variety, and the case where direct application is possible is rare. Also, companies are often seeking direct solutions. Indirect practices involve the search of causes, the pursuit of solutions, and the final attainment of solutions, but the case is often, "the situation is this right now, and we need a solution right now," without the search of the cause. Companies often see a phenomenon as simple saying, "it was running well until now" or "it doesn't go well for new products," and is often led to thinking that "there is no problem in the way we use to do it" or "the bad part is only this (the actual trouble) and all will be well if this is fixed." However, the reason "it was running well until now" is because the conditions were good or they were "lucky." Particularly for manufacturing technology that existed for a long time, the difficulty increases daily, and many companies may not be aware of this. In the most advanced fields, work is being done at levels that are not written in text books, and the way of thinking about the issue is different. When one does not realize that things are becoming difficult, the consciousness turns to "it must go well," and fails to seek the essence. In the joint research with Komatsuseiki, the experience where "all went well" worked against the situation and the cause of the real problem could not be seen. AIST researchers worked to find the cause of the problem by analyzing the reasons why things did not go well one by one, without relying on the newly made technology. As a result, the solution of the problem was obtained, and the company attained consciousness for "looking at more difficult issues" and "seeking solutions at a higher level" through the process, and built the strong will to engage in the search of the cause. In this joint research, at the beginning of the development, the question "isn't there a problem in this area?" was brushed off with "there was no problem there before." However, the cause was finally found by clarifying the individual factors of the issue. In retrospect, I think this detour was a necessary process by which the company and the research institution found common ground, and it can be said that the detour became the foundation for tackling the next issue and more difficult products. From the last fiscal year, we started tackling the joint industrial support theme, and we are engaging in difficult, high-level technological development that enables the manufacture of micro-precision products that could not be made with conventional technology. The current joint research is for the technology to visualize the invisible process in order to accurately analyze technology. This is like adding new pages to a textbook, but it will increase the knowledge and experience of the engineers and provide easier progression to difficult processing for the company. In the industrial support program, we aim not only for a single company, but mass production through collaboration with multiple companies including the upstream material manufacturers and downstream final product manufacturers, as well as research institutions such as AIST and universities. Since the company has 30 % of the world share, it is trying to win the competition against the world in the next generation, without being afraid of taking a detour. The company must accept taking the long way, and the research institution must be willing to take time to handle matters carefully. As a result, we can get new, difficult, and high added-value products. Although the advancement of manufacturing technology is the basis of creating new industry, time is necessary. It is necessary that a technology be effective in the next development of processing with stricter conditions than the one that is being worked on, and it is also necessary that the effectiveness be visible. Pressworking is a processing technology that has been used since ancient times. Some people think that it is "past technology" and "it is sufficiently handled by companies," and it is declining as a research theme for universities and research institutions. However, the actual corporate production sites are perpetually challenged with new issues. Solutions cannot be obtained simply by "buying new devices" or "buying new technology," and only when the cause is found and a true solution is obtained, one can rise higher. In this sense, the role of a research institution is important.

I hope this paper will give some guidance to the future joint research between research institutions and companies.

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Authors

Takafumi KOMATSU

Graduated from the Department of Mechanical Engineering, Tokyo Denki University in 1995. Studied business administration in Ireland and England from 1995 to 1999. Joined Komatsuseiki Kosakusho Co., Ltd. in 1999. Person-in-charge of Production Technology; Section Chief, Production Management; General Manager, Manufacturing; and currently, General Manager of R&D and Executive Managing Director. Established nano grains Co., Ltd. in 2014 and became President. In this paper, wrote about capturing the specific issues at the site of production and promoting their solution, and the results of collaboration with AIST and the changes that occurred in the company due to such collaboration, from the perspective of person in charge of promoting joint research on the corporate side.

Shizuka NAKANO

Completed the master's program at the graduate school of University of Electro-Communications. Joined the Mechanical Engineering Laboratory in 1989. Dispatched to the New Energy and Industrial Technology Development Organization (NEDO) in 2001. Received Doctor of Engineering from University of Electro-Communications in 2003. Worked on the development of manufacturing technology with emphasis on ion beam processing, Micro Machine Project. Other than the fine shape pressing presented in this paper, currently studies minimal fabrication and metal layer formation technology. Group Leader, Function Forming Group, Advanced Manufacturing Research Institute, AIST. In this paper, worked on the visualization of pressworking, the development of surface treatment punch, as well as carried out the visualization technology and its analysis/evaluation in the joint research with Komatsuseiki Kosakusho Co., Ltd.

Discussions with Reviewers

1 Overall

Comment (Naoki Ichikawa, Advanced Manufacturing Research Institute, AIST)

This paper describes an example of problem solving in micro-piercing for pressworking, and the collaboration between AIST and a small/medium company is described through each other's perspectives. The importance and necessity for regional small/medium companies to collaborate with AIST or universities due to changes in business circumstances are explained. Also, communication between the company and AIST, sharing of awareness of the problem, mutual trust and honest proposals, and suggestion of wide-ranging technologies that may lead to problem solving are described along with the progression of the research topics. It can be seen that the process is important in handing over the technology to small/medium companies.

It is specifically mentioned that there are differences in the ways that researchers and on-site engineers think about problem solving of manufacturing technology, as well as how such

differences are surmounted to form the collaborative relationship to solve the issues. This paper is important as a success story of an approach to transfer the manufacturing research result to the site of production. The point of success in collaboration between researchers and production sites is that the way of thinking for problem solving by the on-site engineers changed as the pressworking process became visible.

2 Initiation and background of the collaboration

Question (Naoki Ichikawa)

The authors describe that the evaluation method and visualization of the phenomenon inside the die as well as punching force measurements were developed in the process of conducting the gold ion implantation experiment, but I think the authors should provide a little more explanation from the company side. It is written that when the experiment was started, the company approved of the point that the evaluation and experiment could be done relatively easily with the existing method, and I believe the company's initial expectation was that the gold ion implantation would extend the lifespan of the punch. However, the experiment did not progress as planned and the gold ion implantation did not do well, yet it was decided the joint research would be continued due to the evaluation results obtained. I would like to know how the company looked at this result, and what was the point that made it decide on the continuation of the joint research.

Answer (Shizuka Nakano)

For the gold ion implantation punch, the situation differed between the laboratory and the production site, and the AIST side was aware from the beginning that direct introduction of technology would be difficult. Particularly, angled micro-piercing was an extremely difficult process that could not be done normally, and it was a difficult research topic, and we were careful in exchanging dialogue sufficiently right from the moment we received the samples. We provided topics for exchange of dialogue, such as having people visit AIST to take a look at our research tools and results, including our main facility that was the evaluation device for the lifespan of die tools. Moreover, we discussed the issues that would be expected when these were brought to the site of production. Since the company plant itself was the aggregation of know-how, we were unable to visit at the time of sample provision in the beginning of research. It was rather difficult for the researchers since we had to entrust the tests to the company. Later we heard that the company had thoroughly tested all methods that it could think of, and was interested in us because we were "developing some weird technology that they had never heard of before." (In fact, ion implantation takes a lot of explanation to gain sufficient understanding.) The fact that the company was relying on the possibility for the "unknown" led to this collaboration.

Also, explanation of the importance of knowing the process and to grasp the situation in the experiment using the provided sample led to the awareness that "phenomena inside the die are really not well known." I think it was good that we landed there. As it is written in the text, I was reminded clearly from start to finish that communication is important.

Answer (Takafumi Komatsu)

For the company, we would obtain the short-term effect if the extension of the punch lifespan was realized by gold ion implantation, but besides that, the management was expecting the long-term effect of generating new ways of looking at things through the collaborative research with AIST. As a result, the extension of the punch lifespan by gold ion implantation was not achieved, but it was clear that new ways of looking at pressworking emerged in our company, and we decided to continue the joint research.

3 Visualization of punch experiment

Question (Naoki Ichikawa)

Since it is difficult to see the die in action, I think the point here is to look at the punch by raising it to the position that it can be observed. From the company side, what was the impression when you saw the changes, and what future possibilities did you perceive from this?

Answer (Shizuka Nakano)

In pressworking, we have always wanted to look at what was going on, but it was something we couldn't see because: 1) if it is made of transparent material, we can only process materials that were weaker than the die, 2) if the process is divided, the condition changes, and 3) if the die is taken apart each time for evaluation, only evaluations for very small number of pressing steps can be done. We roughly understood the situation based on the long hours of research activities by our forefathers. However, due to the advancement in processing, more detailed analysis was necessary, and it became important to seek the essence of processing such as for micro-piercing and complex shapes for which solutions could not be found by past theories. Therefore, the importance certainly increases for technologies that enable seeing or estimating what cannot be seen. In the lifespan evaluation device, we were not looking at the moment of pressing, but were able to build a system for observing the tool surface for each piercing, and this was realized at the actual production level although it was a little slower at 40 SPM (shots per minute). It achieved the level that allowed a simulation test of an actual pressworking plant. Moreover, it is important to capture "the abnormalities that occur sometimes" and to review them by repeating similar experiments several times. The "phenomena that occur all the time" are issues that are easy to find solutions for, but "phenomena that occur sometimes" are difficult to grasp, and are actually the biggest problems at the site of production. Since they are probabilistic phenomena, it is considered that to run the process a number of times is the only way to get to the problem. However, we were able to create a technology to catch and hold this slight probability, and this is the point that matched the company's demand.

Comment (Takafumi Komatsu)

The company side was thinking that it was impossible to see the changes in tools after each punching process, but that assumption was overturned in this research. What we could not see became visible, and we expected new impact at the site of production. As a result, understanding deepened at the site of production as the phenomenon was broken down and the measures were taken according to the base cause. That matched the initial expectation.

Question (Naoki Ichikawa)

The authors state clearly that "there was no research or case study," but I think it would be better to consider using the expression "such work has not been done." In fact, I think there are studies that evaluate each shot, though not for micro-piercing.

Answer (Shizuka Nakano)

For example, a method of evaluation after 1,000 shots where the die was disassembled had been done at the Mechanical Engineering Laboratory. This was punching for holes with a diameter of 10 mm. The issue in this case was since the die was disassembled, the same condition could not be reproduced after the die was reassembled. Also, I have not found evaluation for shot-by-shot even for micro-piercing of 10 mm class. The reason is fairly clear, as certain conditions are needed for pressworking under strict conditions that is equivalent to the stress on tools for micro-piercing. One is the case where the plate thickness is thicker than the hole diameter. This is the case where an 8 mm diameter hole is punched into a 10 mm plate, but there are very

few examples. The next condition is the processing precision of the tools, but in the current situation, the processing precision of the tools is not different for micro-piercing and normal size piercing. That is, micro-processing must be done at a condition with relatively low precision. The processing tolerance for the tool in the case where a 8 mm diameter hole is punched into a 10 mm plate can be realized at 1 μm. With tight clearance of 5 %, there is a gap of 0.5 mm (500 μm), and processing can be done by combining the tools with processing tolerance of 1 μm. In this case, it is clearance: tool precision = 500:1. On the other hand, the processing tolerance is about 1 μm for micro-processing. Since the 5 % clearance for plate thickness 0.1 mm is 5 μm, it is clearance: tool precision = 5:1. Since sufficient precision is not attained in the current micro-pressworking, the issues of lifespan and others show up clearly. On the contrary, for macro-processing, relatively high-precision tools are used, whereby the wear and adhesion become relatively small, and it was not necessary to thoroughly consider the lifespan issues. In experiments at ordinary scale, the effect of one shot is small, and there was no need to follow the change after every shot. In micro-processing, the effect of one shot is relatively large, and our research was necessary.

4 Points for successful collaboration

Question (Naoki Ichikawa)

Please organize the points for successful collaboration and the specific course of development. Please present clearly what goals and which actions were taken at each step of evaluations and reviews at the company side, and how the differences in thinking, such as the opinions of the partners, were overcome (or how compromises were made). You say that you obtained results that satisfied the company by visualizing the fine changes in conditions using the observation device. How did you fill in the gap between what the company was demanding?

Answer (Shizuka Nakano)

What was most effective, I think, is that we gradually came to an agreement through exchange of opinions done every month during the experiments at AIST. Since the experiment involved repetition and the experimental device was automated, we set and started the device, and we exchanged opinions while watching the image or the load data as the experiment ran. If there were abnormalities during the experiment, we considered the cause, and if there were no abnormalities, the experiment ran on, and we talked about the expected causes and other topics. In doing so, the thinking that seemed to be quite apart came closer together, and as a result, we reached the point of "how to evaluate the issues at the site of production." Although the details cannot be presented because it is the actual know-how, for the final on-site evaluation, I made a rather difficult request to the workers at the plant. Since the person-in-charge at Komatsuseiki could take responsibility and appreciated the value of carrying out the experiment, it was done, the final proof of cause was confirmed, and this led to the result. I think this was achieved by us going about "stubbornly," "never giving up," and by "not jumping to conclusions."

Comment (Takafumi Komatsu)

In joint research between small/medium businesses and research institutions, in many cases, the research is narrowed due to the focusing of the issue. This tendency is strong particularly in the joint research with universities, and there is a tendency to set one solution per one issue. However, in the research with AIST, solutions are obtained through multiple, comprehensive methods that are studied concurrently. In a small/medium company, since one person must cover much ground, research that runs close to the site of production is necessary as in this case.

Question (Seigo Kanemaru, Electronics and Manufacturing, AIST)

To understand the points of successful collaboration in this paper, please consider the following. In working on visualization, what kind of discussions did you have with the on-site engineers on what to make visible, and what kind of alterations did you make to the evaluation device? As a result, you mention that the pressworking cause-and-effect diagram was refined and this was effective in improving the production procedure. What were the reasons that enabled the refinement of the cause-and-effect diagram? If you have a specific example of such a diagram that was applied to on-site activities, I think it will further clarify the effect of visualization.

Answer (Shizuka Nakano)

For the on-site engineers, we started with the points, "what can be seen in this research" and "what is actually seen on site." In fact, it's "there are things that cannot be seen in research" and "people on site think they know it all," but we had people understand the meaning of each individual data, the meaning of change of load in the interim process, as well as the point that a process that seems to happen in only a few milliseconds is actually separable into multiple processes and that their situations change from moment to moment. The initial evaluation device was slow, and a camera was on one side only, but for the research with Komatsuseiki, cameras were installed on both sides and high speed was achieved. The production speed of pressworking is fast, and high speed was necessary to understanding the phenomena occurring there, and cameras on both sides were necessary to ensure there were no missed shots. Of course, there is progress between the first experiment and the latest one, and the complexity has increased considerably. There has been evolution where the level of processing that could not be achieved at the start of research is now being done normally at the production level, and this has become possible because we understood the phenomena well and the problems were captured accurately.

Advanced ignition technology for the achievement of high thermal efficiency of internal combustion engine

— A demonstration of laser ignition in natural gas engines —

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Natural gas engines have been attracting a lot of attention recently due to the development of unconventional natural gas sources. Achieving lean burn with supercharging is necessary to attain high thermal efficiency. Conventional spark plugs face difficulties with ignition because of the high pressure and lean air/fuel mixture. This paper describes studies on laser spark ignition which has been investigated at AIST as an alternative method for the achievement of stable ignition under such conditions. The extension of lean limit and improvement in thermal efficiency are demonstrated, and the possibilities of advanced laser ignition are also discussed.

Keywords : Natural gas, lean-burn, supercharging, laser ignition, thermal efficiency, cogeneration

1 Introduction

Unconventional natural gas resources have been developed recently all over the world because of advances in mining technology. The total natural gas reserve including reserves present in unconventional mines is estimated to exceed 200 years, and these mines are distributed globally.[1][2] Compared to the usual liquid fuels, natural gas has higher hydrogen-to-carbon ratios in its molecules, and it produces fewer carbon dioxide emissions per unit of calorific value. Furthermore, the sulfur content in natural gas is very small, and it has therefore attracted interest as fuel for marine engines to enable them to adhere to recently developed exhaust gas regulations.[3] Therefore, the role of natural gas is expected to increase in importance as a major energy source.

Natural gas is also used as a fuel for cogeneration (combined heat and power), which produces not only electricity but also heat. Therefore, its total efficiency is high. However, according to a report published by the American Council for an Energy-Efficient Economy (ACEEE) titled "The International Energy Efficiency Scorecard," Japanese buildings have a low energy-utilization efficiency and the use of cogeneration is little.[4] Therefore, cogeneration is expected to improve this situation.

There are several types of cogeneration alternatives, such as fuel cells, gas turbines, and gas reciprocating engines. Gas engine cogeneration ranges in scale from being compact for family use to large for industrial applications, and there is therefore a large number of installations. The electrical power efficiency of the latest gas engine was a lower heating value (LHV) of almost 50 %. This is because of the application of technologies such as lean burn and mirror cycles.[5]

Even though cogeneration has a high total energy-utilization efficiency, the practical application of conventional cogeneration is suitable for cases that require a relatively large amount of heat because the proportion of heat obtained from cogeneration is still large. In general, electricity is a preferred product than thermal energy. Therefore, it is important to increase the thermal efficiency for electrical power generation to accelerate the adoption of cogeneration technology.

2 Technological issues affecting natural gas engines

2.1 Thermal efficiency of gas engines and future direction of technological development

The improvements in the thermal efficiency of gas engines are important for the promotion of gas engine-based cogeneration installation. Obviously, the maximum thermal efficiency is limited by the Carnot cycle according to thermodynamics. The actual gas engine is an irreversible engine, and the Otto cycle model, which is closer to a real engine, is used to understand its fundamental behavior. In the Otto cycle, fuel is supposed to burn at the location of the piston's top position, which corresponds to the maximum compression. On the contrary, it is supposed to be exhausted at the bottom piston position, which corresponds to the maximum expansion. The thermal efficiency of the Otto cycle η_{th} is expressed in the following equation:

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$$\eta_{th} = 1 - \left(\frac{1}{\varepsilon}\right)^{\kappa-1}$$

Here, ε is the compression ratio, and κ is the specific heat ratio, which is the ratio of the specific heat at constant volume and constant pressure. According to this equation, the relation between the thermal efficiency and the specific heat ratio is as shown in Fig. 1. As can be seen, the higher the compression ratio and the specific ratio, the higher the thermal efficiency becomes. With respect to the compression ratio ε, even though the natural gas is less likely to realize abnormal combustion, too large compression value leads to greater heat loss and/or abnormal combustion; thus, the practical value of the compression ratio is limited to around 14.

On the other hand, each molecule has its own specific heat ratio. Nitrogen and oxygen molecules, which are major constituents of air, are both diatomic molecules, and these have three translational degrees-of-freedom and two rotational during motion. The specific heat ratio of these is κ~1.4 around room temperature. Fuel molecules such as methane are polyatomic molecules. The specific heat ratio is around 1.3 because the molecules have more degrees-of-freedom during motion. Therefore, the total specific heat ratio of the air/fuel premixture is determined by the mixing ratio of air and fuel. Thus, we can increase the specific heat ratio by leaning out the premixture. Furthermore, the specific heat ratio decreases with increasing temperature because of the increase in the number of degrees-of-freedom, which better distributes the heat energy. Decreasing the temperature by the lean combustion is also effective to maintain a high specific heat ratio.

Thus, lean combustion and exhaust gas recirculation (EGR) are used to decrease the combustion temperature in order to improve the thermal efficiency. However, the consumption of fuel for each cycle decreases by simply leaning out the mixture, and the output power decreases. In order to compensate for this, turbo boosting is also employed. Recent compression end pressures that have been realized are almost 10 MPa (almost 100 atm). The recent technological trends in brake mean effective pressure (BMEP) and equivalence ratio is shown in Fig. 2. The equivalence ratio $\phi = 1$ corresponds to the stoichiometric reaction of fuel and oxygen in air. A major trend of the technology is leaning out and boosting out to increase the BMEP. This increase in the BMEP also causes knocking, and leaning out is likely to result in misfiring. Thus, it is necessary to go through the "corridor" between the knocking and misfiring. Ignition devices have to realize stable ignition under this high pressure and lean premixture.

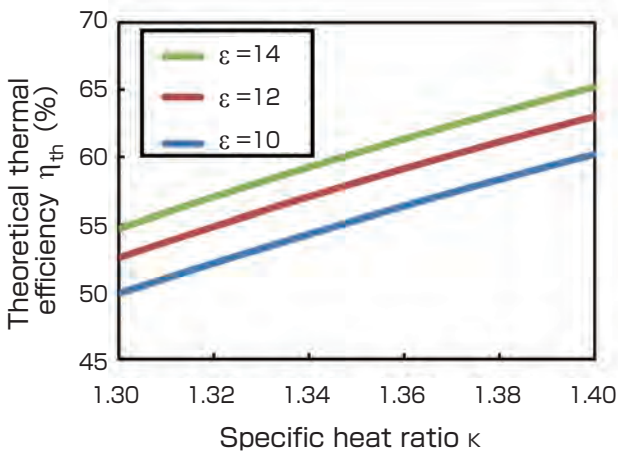

Fig. 1 Relationship between the specific heat ratio and the thermal efficiency of the Otto cycle

2.2 Problems of conventional ignition method: spark plug

The commonly used electrical spark ignition method is an ignition method that was invented more than 100 years ago. In spark plugs, discharge plasmas are formed between the high voltage and grounded electrode, and this causes ignitions to take place. Even though there have been continuous efforts to improve spark ignition, spark plugs are approaching their technological limit based on recent technological trends that see high boosting and lean burn.[6]

To generate discharge plasma, it is necessary to increase the number of electrons between the electrodes by accelerating them to form electron avalanches. Electrons in air obtain energy by accelerating in the mean free path of surrounding neutral molecules. These accelerated electrons collide with neutral molecules, thereby ionizing them. As the number density increases, the electron mean free path also shortens. Thus, to have sufficient ionization energy, the strength of the electric field also has to increase to compensate for it. In other words, in principle, the discharge is scaled by E/N (E: Electric field strength, N: Number density). Considering

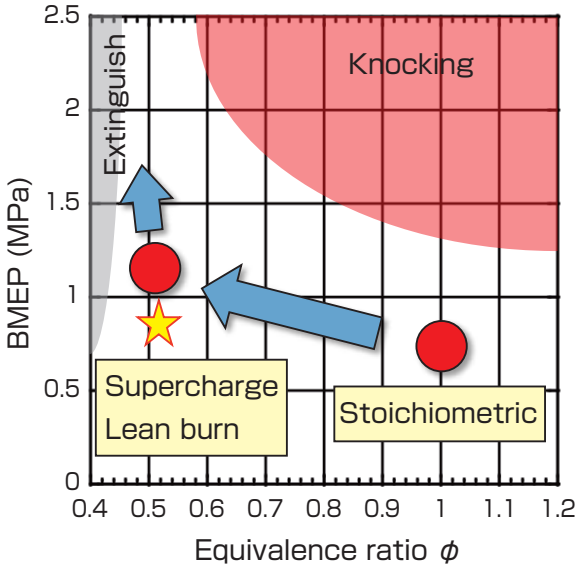

Fig. 2 Technology trends expressed in output as brake mean effective pressure and the equivalent ratio of the premixed gas

the supercharging of engines in the near future, this is a disadvantage for conventional spark plugs because it is necessary to increase the discharge voltage, which will cause not only shorter plug lifetimes, but will also lead to problems related to the dielectric insulation in many parts.

2.3 Alternate advanced ignition technology: laser ignition

Research that involves the use of pulsed laser for the ignition of combustible mixtures has been conducted in institutes worldwide, including the Mechanical Engineering Laboratory (MEL), which was one of the former institutes of the National Institute of Advanced Industrial Science and Technology (AIST) in Japan.[6][7] Schematics of engines that use conventional spark plug ignition and YAG laser ignition are shown in Fig. 3.

In laser ignition, laser pulses are focused by a convex lens, and plasma is formed by the dielectric breakdown of the premixture, which achieves ignition. Both lasers and spark plug ignition utilize hot plasmas, but the physics behind their formation is different. Therefore, the influence of both ignition methods on future supercharging engines will be different.

Plasma formation in laser ignition goes through the following two-steps (Fig. 4). First, focused high-intensity laser pulses produce electrons in the focal region through multi-photon ionization of molecules, which will be the initial electrons of discharge. Then, these electrons efficiently absorb laser energy through the inverse Bremsstrahlung process.[8] Because these processes are faster in mediums with a larger number density, laser breakdown is easier in supercharged engines. One of the commonly asked questions regarding laser ignition is whether there is a maximum pressure at which laser plasma can ignite. Considering the plasma formation in water, which has a density that is more than 100 times larger than that of ambient air, lasers can form plasmas easily, but electrical discharge requires a higher voltage. This suggests that the pressure is almost no problem for laser ignition. Flame-kernel developments observed by fast cameras using laser and spark plug ignition are shown in Fig. 5. In the upper pictures showing the laser ignition, a donut-shaped vortex is formed and the flame propagates quickly. On the other hand, in the lower spark plug ignition, the flame propagation is slower, which is due to the heat loss to electrodes. In addition, a characteristic vortex, which works as a flame holder, is formed in laser ignition.

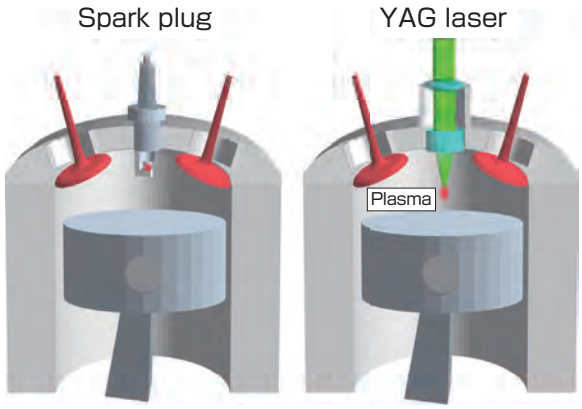

Fig. 3 Comparison between spark plug ignition and laser ignition in reciprocating engine

3 Scenario for the application of laser ignition in engines

In order to develop laser ignition for practical use, it is important to demonstrate the technical advantages, especially in supercharged engines, and the improvement in terms of thermal efficiency. Then, we can evaluate its feasibility based on the technological trends of gas engines. Furthermore, the degree of the improvement in the thermal efficiency enables us to estimate the payback time of any investment in this advanced ignition.

On the other hand, for general use, it is also important to consider the outlook of laser development with respect to the development of more compact, stable, and low cost laser devices. Long-term stability is essential, especially for cogeneration engines, because it will operate continuously for many months. The cost of the laser can be cheaper than that of engines for middle-to-large size cogeneration;

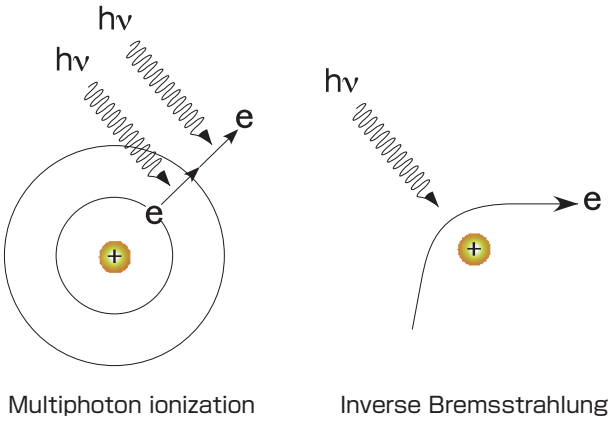

Fig. 4 Laser breakdown formation processes (multi-photon ionization and inverse Bremsstrahlung)

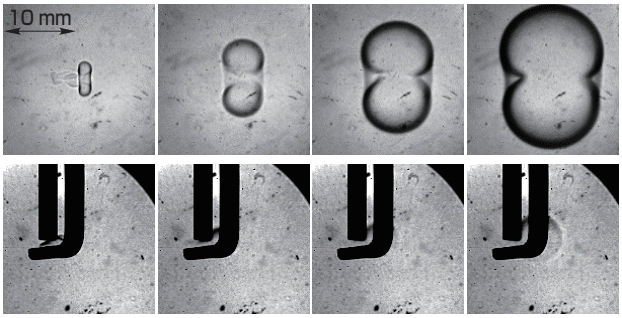

Fig. 5 Temporal flame-kernel development for laser and spark plug ignition

therefore, the cost may be recovered by the reduction in the fuel cost. In any case, as the price of the laser decreases, it is expected that it will be more widely used.

Figure 6 shows a diagram that represents the temporal progress of laser ignition research at AIST, the development of non-conventional natural gas resources and gas engines, and the revolutions of laser technology. This diagram also shows the outlook of laser ignition considering the relation between problems and progresses. Individual technologies and details of the demonstration experiments will be described in the following sections.

3.1 Innovation in the development of compact laser devices

Laser ignition research has been conducted at AIST since the days of the former research institute, MEL, and included laser ignition using photochemical reactions[9][10] and dielectric breakdown formed by laser pulse focusing.[11][12] For practical use, both a demonstration of the advantages of laser ignition, which has been achieved partly at AIST, as well as the realization of several innovations using laser devices are required. Because conventional lasers are precision instruments, they lack stability and durability and moreover, they are expensive as ignition devices. Therefore, improvements in the energy efficiency and possible continuous operation time of laser systems were indispensable. In this regard, replacing conventional flash lamps for laser excitation with laser diodes enabled more long-term operation, which also improved the energy efficiency from less than 1 % to around 10 %.

Next, an important issue to be resolved was the long-term stability and outlook with respect to reduced cost. These problems were solved by fabricating microchip composite lasers.[13][14] Conventional solid-state lasers used laser materials that are obtained by crystal growth. However, it was demonstrated that ceramics created by sintering can be used as optical material by sufficiently increasing the density to suppress the scattering from the grain boundaries. Furthermore, Taira developed a composite ceramics laser that contains a laser-medium, saturable absorber for Q-switch operation, and a mirror. A picture of the laser is shown in Fig. 7. A comparable laser size relative to that of spark plugs

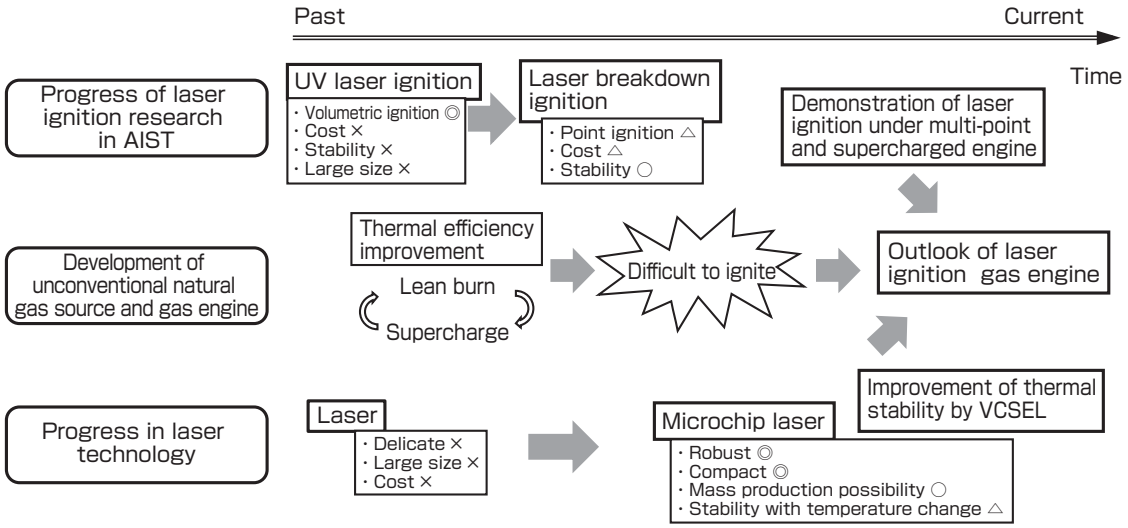

Fig. 6 Scheme and relationship of time evolution for each technology related to laser ignition of gas engine

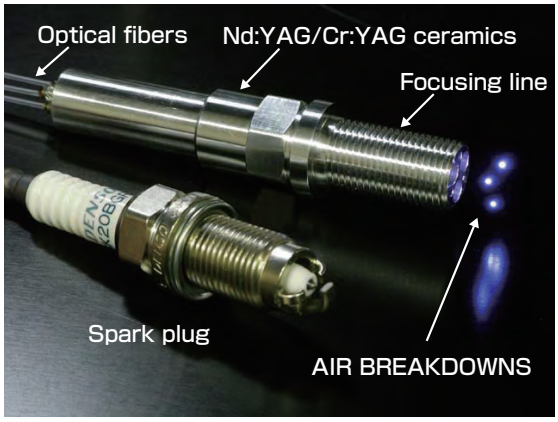

Fig. 7 Microchip laser[14]

Table 1. Specifications of the test engine

Base engine	NFD170
Type of engine	Four stroke
Bore × stroke (mm)	102×105
Displacement (cm^3)	857
Compression ratio	12,14
Fuel	Methane
Rotation speed (rpm)	1200
Ignition timing	MBT
Swirl number	2.15~2.45
Maximum pressure (MPa)	8

has been realized. Innovations such as miniaturization and integration have increased the attention on laser ignition as a feasible technology.

3.2 Demonstration of laser ignition advantages in highly supercharged gas engines

It has been necessary to demonstrate the advantages of laser ignition over conventional spark plugs for recently developed supercharged gas engines. Here, we introduce the experimental results obtained by joint research between AIST and Mitsui Engineering & Shipbuilding Co. Ltd.

Figure 8 shows the experimental layout of the demonstration experiment involving a gas engine. We used a modified diesel engine, and compressed air was introduced from the compressor. Other parameters of the engine are listed in Table 1.

Because the maximum cylinder pressure of the engine is 80 atm., the intake-supercharged pressure was limited to 1.8 atm. The main result of the demonstration, which is the relation between the indicated mean effective pressure (IMEP) (not including mechanical losses) and the equivalence ratio, is shown in Fig. 9. The horizontal axis represents the equivalence ratio and the left side of the figure is leaner. The vertical axis corresponds to the output from the engine. The spark plug and laser ignition results are represented by square and circle points, respectively. Data in normal aspiration, without supercharging, are indicated as hollow characters, and the colors, which are listed as a table in the figure, represent the intake pressure. For normal aspiration experiments, the laser results maintained higher IMEP compared to spark plug ignition. Then, for the case of supercharging, spark plugs that are represented as red squares rapidly shifted toward the rich side. This indicates that spark plugs cannot ignite for the premixture under high-pressure conditions.

On the other hand, it has been demonstrated that lasers can maintain stable ignition even in the supercharged condition up to an intake pressure of 1.8 atm., which is the limit of the engine system.

3.3 Demonstration of improvement in thermal efficiency due to implementation of multi-point laser ignition in gas engines

In order to demonstrate the advantage of thermal efficiency improvement resulting from laser ignition, we conducted a dual-point laser ignition experiment using the same gas engine. The effect of the ignition on the coefficient of variation (COV) of the IMEP and indicated thermal efficiency are shown in Figs. 10 and 11, respectively. As can be seen in Fig. 10, the spark plug ignition rapidly becomes unstable below the equivalence ratio of 0.63 because of the unstable ignition. The lean burn limit is extended using laser

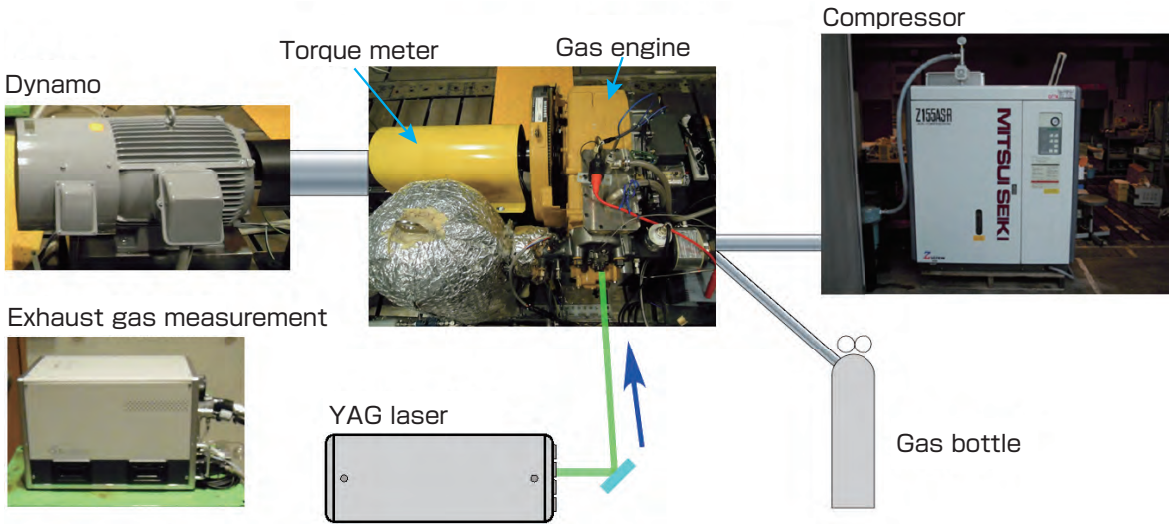

Fig. 8 Layout of demonstration experiment of laser ignited gas engine

ignition, which is represented as a red curve. Furthermore, the stable operation region of the equivalence ratio is also enlarged by the dual-point ignition.

A similar improvement is also observed in the thermal efficiencies. The dependence of the indicated thermal efficiency in Fig. 11 shows the improvement in all ignition methods when it is leaned out. However, for the case of the spark plug, a steep degradation was observed at an equivalence ratio of around 0.63, and this was due to the unstable ignition. On the other hand, for the laser ignition extension of the lean burn limit, the thermal efficiency increased. The main reason for this improvement is the fast formation of the initial flame kernel, which is attributed to the absence of heat loss to the electrode for laser ignition. Meanwhile, after the initial flame-kernel development, there is no difference between the single laser ignition and spark plug ignition methods with respect to the duration of the entire cylinder burn. Furthermore, double laser ignition achieved a shortening of the burning time after the initial kernel formation. This is due to the increase in the flame area, which enables an increase in the thermal efficiency.

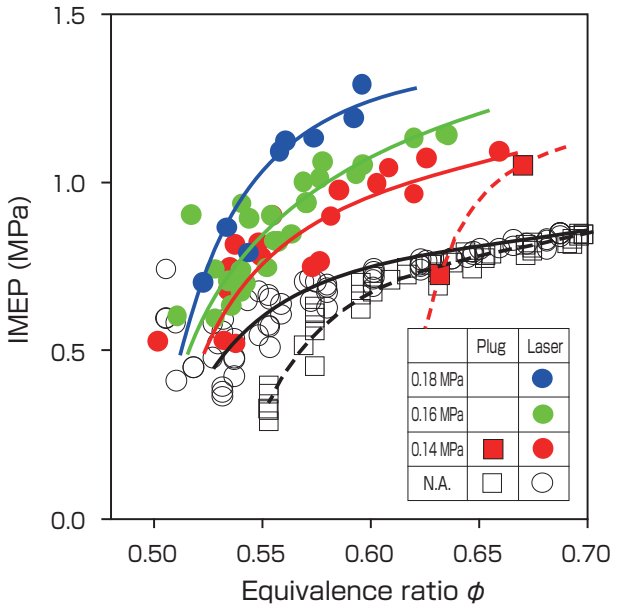

Fig. 9 Relation between the equivalence ratio and indicated mean effective pressure (IMEP) under the supercharging condition

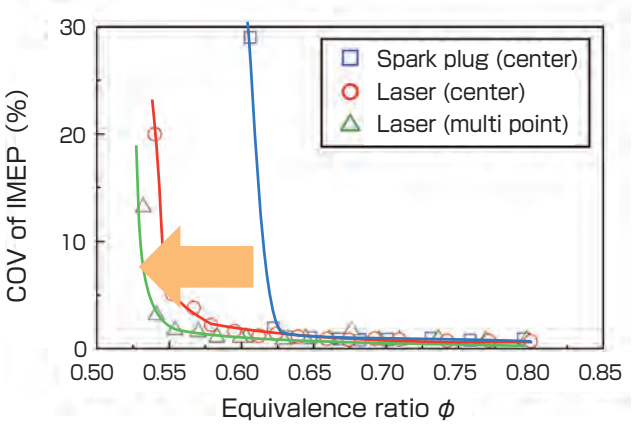

Fig. 10 Dependence of COV of IMEP on equivalence ratio

3.4 Exploration of advanced laser ignition for popular use

The required technologies for the application of laser ignition are listed in Fig. 12. Even though we aim at the use of laser ignition in large gas engines, the cost of the laser remains an important obstacle. Besides, there are many gas engine sizes, and the number of cylinders varies. There is a large cost difference depending on whether we can deliver the output of a laser system to every cylinder, or whether each cylinder requires a laser to ignite. The cost of current pulse lasers is of the order of several million yen, and this needs to decrease by several orders of magnitude. Meanwhile, the microchip ceramics laser that was mentioned in the former section is suitable for mass production, so a significant decrease in cost is expected if the market for the laser ignition increases with synergetic influences. Nevertheless, efforts to reduce the cost and improve the durability by reducing the required specifications are important, especially with respect to pulse energy. A further extension of the lean burn limit is still important. Therefore, we have to return to basic science to examine the breakdown process of the laser itself.

As described in a previous section, laser breakdown is caused by the multi-photon ionization and successive laser energy absorption through the inverse Bremsstrahlung process. Multi-photon ionization depends on the power of the

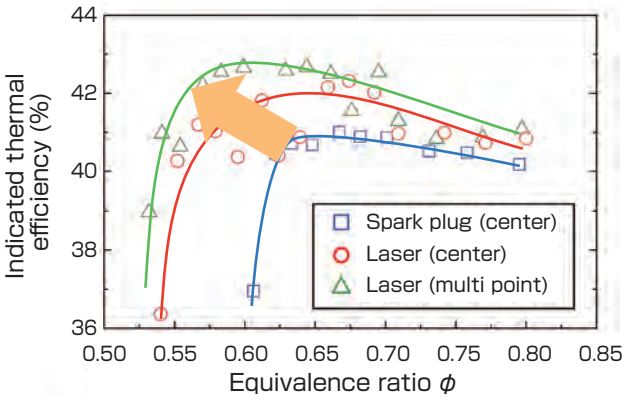

Fig. 11 Improvement of indicated thermal efficiency due to multi-point laser ignition

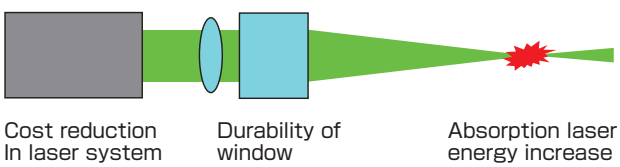

Fig. 12 Necessary technology for the development of laser ignition technology

focused laser intensity, so plasma formation is significantly affected by the laser intensity. The microchip-ceramics laser generates laser pulses with sub nanosecond pulse widths by employing short cavity Q-switch operation using a saturable absorber, and it generates laser pulses with high-peak power. The energy that is required to achieve ignition is obtained by the multi-pulse incidence. On the other hand, a common Nd: YAG laser generates laser pulses with pulse widths of several nanoseconds, which offers sufficient energy for easy ignition. However, the efficiency of utilizing laser energy for ignition is not very high because the intensity of the rising edge of the pulse is low and the multi-photon ionization is small. Here, we introduce several fundamental attempts to overcome these issues.

In order to separate the multi-photon ionization and inverse Bremsstrahlung processes, initial electrons were separately introduced in arbitrary time and space in order to observe the effect on the ignition. We used a Ti:Sapphire (TiS) laser, which can generate laser pulses with femto-second pulse widths, to generate the initial electrons. The pulse width of the TiS was 150 fs, so the peak power reached 1 GW even though the energy of the pulse was only 100 μJ, and we can use it to realize microlaser breakdown, which by itself cannot ignite the premixture alone. As shown in Fig. 13, to evaluate the influence of initial electron seeding, the TiS laser pulse was injected orthogonally to the YAG laser for ignition.

The absorption of the YAG laser was significantly influenced by the seeding, as shown in Fig. 14, which shows the relation between the incidence and absorption of the laser. The YAG laser requires around 35 mJ of energy to generate breakdown due to the pulse alone. By suppling the seed electron, the threshold decreased and the main YAG laser energy was absorbed very efficiently. Even for the lowest energy, the data point of the YAG energy for the dual laser in Fig. 14 was able to ignite the premixture.[15]

Images of the breakdown processes observed by a gated-intensified CCD camera are shown in Fig. 15. These pictures were taken with a 5-ns gate time and at 5-ns intervals. The relative position of the YAG laser-focusing beam and the TiS laser are in the leftmost picture. The TiS laser was focused at almost the center of the focal point of the YAG laser. The YAG laser pluses below the threshold of its breakdown alone were able to start breakdown by creating a slight ionization location. We also observed ionization wave propagation. The propagation velocity was estimated to be 10^5 m/s.[16]

Thus, we found that the YAG laser energy for the ignition may be significantly reduced by supplying initial electrons. In addition, the dependence of the absorption energy in Fig. 14 both with or without TiS have almost the same gradient over the range of energies of the incidence laser, which suggests the existence of an additional energy loss channel in the case without seeding electrons. Therefore, the lean burn limit can be extended using this method. However, it is not so practical when using the TiS laser, which is very complicated and delicate for ignition; therefore, we require simpler methods to supply electrons. One possibility is the combination of ultra-violet lasers, which merits further study.

In addition, the durability of the window for the laser incidence is another important technical issue to be resolved. Several papers have reported the accumulation of soot or deposits on the window, while others report the self-cleaning effect of lasers by the incidence of laser pulses with high fluence, which burns out the soot on the window or maintains the temperature of the window at a sufficiently high value to prevent the accumulation of deposits on the windows. In the experiments performed by our institute this phenomenon was not observed, but several potential reasons may be considered. Because the mechanism of its formation and

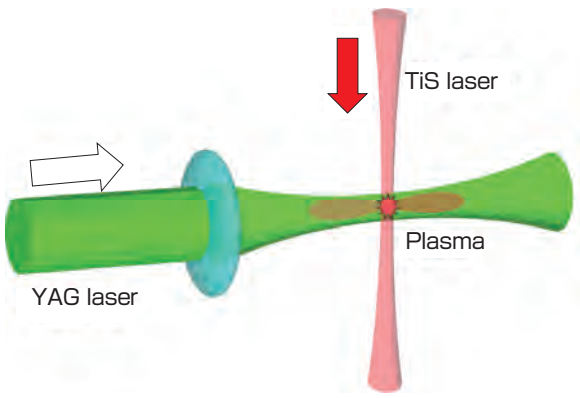

Fig. 13 Breakdown process of YAG lasers with initial electron

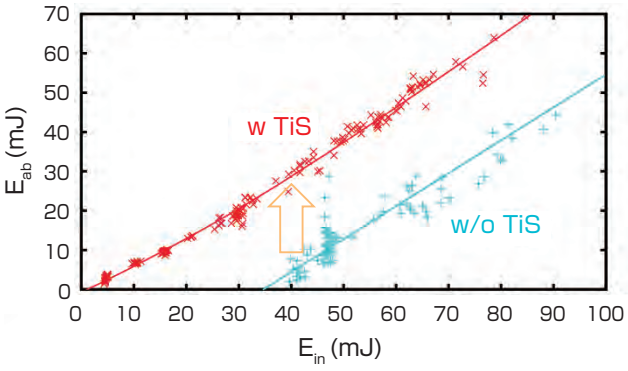

Fig. 14 Effect of TiS laser on the absorbed energy (E_{ab}) of YAG laser vs incident energy of the YAG laser (E_{in})

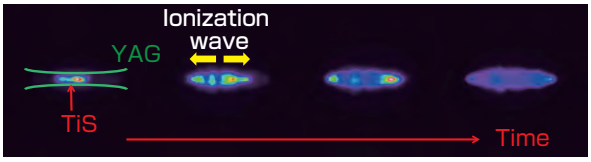

Fig. 15 Temporal breakdown process with initial electron supply

suppression methods is not established, it is necessary to carry out systematic research in this area.

In this review, we discussed the potential for the application of laser ignition for gas engines. The market for automobile engines is so large that the impact will be large if the ignition method is commonly used. However, there is a greater requirement for laser systems such as cost, size, and long-term stability. The potential for using a vertical-cavity surface-emitting laser (VCSEL), which has excellent temperature stability at its oscillation wavelength for the pumping of the microchip ceramics, was recently demonstrated.[17] It is expected that the use of laser ignition will be first applied to the cogeneration market, after which cost reductions will lead to development in other markets.

4 Summary

Because of the inherent advantage of laser ignition in high-pressure environments, it is attractive as an alternative ignition method for lean-burn supercharged gas engines. The innovations regarding laser systems, particularly with respect to its compactness, the use of composite configurations, and stability enable the widespread application of this method. In such an environment, demonstration experiments that show the advantage of laser ignition compared to conventional spark plugs have been conducted at AIST. In order to realize a more widespread acceptance and use of laser ignition, there is a need to realize a reduction in cost; therefore, we examined the potential for the application of the advanced laser ignition method, which was obtained from our fundamental investigations.

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Received doctorate degree in physics from Tsukuba University in 1994. Joined Electrotechnical Laboratory as researcher, where he engaged in laser fusion research. He has been involved in the application of laser ignition to internal combustion engines since 2009. His current research interests relate to the application of plasma to internal combustion engines, including laser ignition. In this paper, he demonstrated dual-point laser ignition and carried out experiments into the fundamental laser breakdown processes.

Hirokazu KOJIMA

Completed the doctorate course in energy science at the Graduate School of Energy Science, Kyoto University, in 2012. Since 2012, he has been a researcher at AIST. He has been engaged in research for engine combustion and highly efficient production and utilization of hydrogen energy carriers. In this study, he investigated the breakdown using a combination of a YAG laser and a TiS laser.

Hirohide FURUTANI

Joined Mechanical Engineering Laboratory at AIST after completing the doctorate course at the University of Tsukuba. He has studied laser ignition and control techniques for engine combustion for over 20 years. His interest is in realizing practical applications of laser ignition by carrying out more research and development into both engine combustion and laser technology. In this study, he performed experiments into supercharged gas engines, and showed the advantages of laser ignition.

Discussions with Reviewers

1 Overall paper

Comment (Akira Yabe, New Energy and Industrial Technology Development Organization (NEDO))

This paper discusses supercharging and lean burn as technological trends related to gas engines, and showed the advantages of laser ignition over the conventional spark ignition as a solution, together with a demonstration. The characteristics and effectiveness of laser ignition are also described systematically, and solutions to issues are offered. I think it is suitable for *Synthesiology.*

Comment (Hiroyuki Niino, AIST)

This paper synthesiologically describes the attempt to employ laser ignition as a substitute for conventional spark ignition to improve the thermal efficiency of internal combustion engines. In addition, the level of the research is high. I think it is suitable for *Synthesiology.*

2 Temporal arrangement of description of research progress in the paper

Comment (Hiroyuki Niino)

In each subchapter, technical obstacles are fully and clearly described. During its development, I assume there was technical progress from the initial stage, as associated technologies, equipment, and feedback from experts in other fields were applied. Therefore, to help the readers' understanding, it is preferable to consider the "synthesiological" scheme of the technological development including its differences with past, current, and future technologies including breakthroughs and serendipity.

Answer (Eiichi Takahashi)

Based on the suggestion made by the reviewer, we have included Fig. 6, which represents the progress of the entire combination of technologies. We hope that the demonstration experiment of laser ignition at AIST, as well as breakthroughs in laser technologies will contribute to overcoming obstacles in the development of future gas engines.

3 The dependence of thermal efficiency on ignition method

Comment (Akira Yabe)

Research on the performance improvement of spark plugs for future lean burn operation will continue. However, it is difficult to evaluate the technological advantages quantitatively although it can be assumed that there is small heat loss to the electrode and flame-holding by the vortex is effective.

It is desirable that this paper explains the following mechanisms. In Figure 11, for the thermal efficiency of spark plugs, lasers, and dual point laser ignition, the results should be on the same line after the equivalence ratio of 0.7. If there are possibilities of differences in thermal efficiencies, the mechanism or reason should be explained. Moreover, the reason for which dual point laser ignition provides better thermal efficiency than single point should be mentioned, at least qualitatively.

Answer (Eiichi Takahashi)

We thank the reviewer for that comment. As noted by the reviewer, the performance of spark plug ignition has continued to improve. By controlling the flow around the spark plug to lengthen the spark channel, we can suppress the heat loss to electrodes, as demonstrated by the laser ignition, and improve the lean ignition capabilities. However, the pressure will increase in the future because of the physical difference in the plasma formation, as described in the paper, and laser ignition has a clear advantage.

The reviewer expected to achieve asymptotic behavior on the spark plug and laser for an equivalence ratio of more than 0.7. We examined the temporal variation of the mass burning ratio, and the initial flame-kernel development is faster for laser ignition even at an equivalence ratio of 0.8. This, we think, brings about the difference in thermal efficiency. With respect to the difference between single and dual point lasers, flame area is double with the dual point than the single-point, and this contributes not only to the period of the initial flame-kernel formation, but also to the successive flame development thus shortening the combustion period. These mechanisms are added in the paper.

4 Progress of laser ignition research

Comment (Hiroyuki Niino)

Please consider the peripheral technology and research, which are necessary for research in the area of laser-ignition technology. Compared with the scenario above in Discussion 2, please indicate the remaining research issues that are required to fill the gap between the current and future technologies. These topics will be better understood if they are presented as a chart.

Answer (Eiichi Takahashi)

The most important obstacle for the practical implementation of laser ignition is the need to reduce the cost of lasers. We have added Fig. 12 to identify the research issues. We described a new method that was used to increase the fraction of laser energy absorption.

5 Spread and installation of laser ignition technology

Comment (Hiroyuki Niino)

The authors claim that the cost of lasers is most important for the widespread use of laser-ignition technology in society. Please discuss the prospects based on laser cost. Moreover, are there other similar issues to be solved? If so, please discuss them.

Answer (Eiichi Takahashi)

The cost of the laser depends on the gas engine for cogeneration, which varies widely, and it also depends on its usage, e.g., whether one laser is used per cylinder, or the pulses are delivered from one laser. The cost is expected to decrease from the current value of several million yen to several hundred or several tens of thousands of yen. Furthermore, the accumulation of soot or deposits on the laser window is another important issue. We have added a discussion about the cost and durability of the window.

Development of a rapid analytical system for glycans using a multistage tandem mass spectral database

— Toward an era where everyone can analyze glycan structure without specialist knowledge —

Akihiko KAMEYAMA[1*], Norihiro KIKUCHI[2], Shuuichi NAKAYA[3] and Shinji FUNATSU[3]

[Translation from *Synthesiology*, Vol.8, No.4, p.200-213 (2015)]

Conducting glycan analysis requires expertise. This requirement has been a major bottleneck in the progress of glycomics. If glycan analysis can be done easily and rapidly without specialist knowledge, then the development of glycan functional analysis and associated applications is expected to accelerate. Here, we describe the construction of a multistage tandem mass spectral database, and a system for rapid glycan analysis that utilizes this database, as examples of infrastructure development for the advancement of glycoscience.

Keywords : Glycomics, mass spectrometry, database, structural analysis, algorithm, library, isomer

1 Introduction

Many readers may not be familiar with the term glycotechnology (or glycoengineering). In fact, already a quarter of a century has passed since this term was born. When genetic engineering and protein technology that were based on the science of nucleic acids and proteins started to have large impact on society as biotechnology, the lack of knowledge of glycans, which is the third chain of life, became a problem. Against such a background, the academic discipline of glycobiology emerged, and the concept of glycotechnology was born in Japan, with the perception that this knowledge should be actively utilized in the field of biotechnology. Details are described in the beginning of *Tosa Kogaku* that was published (by Sangyo Chosakai) in 1992.[1] After about 10 years, the "Glycogene (GG) Project" was started by the New Energy and Industrial Technology Development Organization (NEDO), followed by the "Structural Glycomics (SG) Project" and the "Medical Glycomics (MG) Project." These glycan projects were led by Project Leader Hisashi Narimatsu for 10 years. The results all the way to product realization of clinical diagnostic drugs are summarized in *Synthesiology* Volume 5 Issue 3 (2012) "Development of basic tools for glycoscience and their application to cancer diagnosis."[2] The first output to society was a clinical diagnostic drug, but the results of this on-going project do not end there. One of the important results was the laying of the foundation for glycan research that was lacking in life sciences, or the infrastructure for "synthesis," "structure," and "function." Glycotechnology that was proposed 25 years ago is taking off now.

The structural analysis of glycans is difficult. The main reason is because, unlike the nucleic acids and proteins for which the primary structure can be known if the sequence is read, there are branch structures, positional isomerisms, stereoisomerisms, and others, and simple sequence decoding is not sufficient to know the glycan structure (Fig. 1). This means that the heart of glycan analysis is how to identify the isomers. The difficulty of glycan structure analysis is described in *Synthesiology* Volume 7 Issue 2 (2014) "Development of lectin microarray, an advanced system for glycan profiling."[3] The glycan structure analysis was a work undertaken by specialists with skilled craftsmanship, and this was a major bottleneck in glycan research. If glycan structure analysis can be conducted easily by anyone, it is expected that the range of glycan research will widen and the clarification of glycan functions that still remain mysterious and their application will rapidly progress. In the SG Project, two approaches were taken for the glycan structure analysis. One is the glycan profiling method[4] where the lectin array, in which various types of proteins (lectin) that can identify the partial glycan structure, is arranged on the slide glass. This method yielded results in the search for disease biomarkers and stem cell markers.[5]-[7] The search for markers demands sensitivity rather than precision, and the highly sensitive lectin microarray that can be prepared easily is utilized effectively. On the other hand, if one wanted to clarify the marker itself at a molecular level or check the content of the multiple glycan types, glycan analysis by mass spectrometry described in this paper is useful. The two methods mutually supplement each other's weaknesses. In this paper, we

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discuss the construction of a multistage tandem mass spectral database as one of the infrastructures of glycan structure analysis and the rapid analytical system for glycans utilizing this database.

2 Glycan structure analysis by mass spectrometry (MS)

Since the development of two soft ionization methods, the electro-spray ionization (ESI) and the matrix assisted laser desorption/ionization (MALDI), the use of mass spectrometry rapidly increased in the life science field. Currently, these are used widely in proteome analysis, pharmacokinetic analysis, biomarker search, microorganism identification, and others. When our research was started in the beginning of the 2000s, the proteome analysis was introduced enthusiastically as the method for post-genomic research, and MS played a central role and evolved rapidly. The glycan structure analysis at the time mainly used the database of retention time for various glycans in the high-performance liquid chromatography (HPLC). However, since precise data could be obtained quickly and with high sensitivity, it was considered a matter of time before MS would replace HPLC in glycan structure analysis. Against such a background, before the start of the project, Dr. Koichi Tanaka of Shimadzu Corporation visited the Research Center for Medical Glycoscience, AIST and introduced the MALDI quadrupole ion trap time-of-flight MS (MALDI-QIT-TOF MS) that was a new mass spectrometer developed in the UK (Fig. 2). With this spectrometer, glycans become singly ionized by MALDI, multistage collision induced dissociation (CID) is accomplished by ion trapping, and resolution is high since it is time-of-flight MS. These characteristics are appropriate for glycan structure analysis where it is necessary to determine the isomers with high sensitivity. Therefore, it was decided in the Project to develop a new glycan structure analysis method using this device.

2.1 Development trend at the beginning of the research

In the precise structure analysis of glycans using mass spectrometer, the method employed was careful analysis of fragment ions obtained by high-energy CID[Term 1] in the fast atom bombardment MS (FAB-MS), after methylation of all OH, NH, and COOH groups of the glycan (permethylation). This method could be conducted by a few, limited specialists of glycan MS, and in the omics[Term 2] boom at the beginning of the 2000s, a new simplified method was sought to replace this method. The method that was employed at the practical level was the creation of a database of a calculated fragment list of all known glycan structures and then referencing the peak list of the MS^2 spectrum of the analyzed glycans to this database. Although this was a simple method, it was not possible to identify isomers that was the heart of glycan analysis. The University of New Hampshire was engaging in research of a method that enabled identification including the isomers, but their method necessitated the permethylation of glycans before analysis.[8]-[10] There was no commercially available device or kit forpermethylation, and it was a difficult derivatization method for those without expertise.

2.2 Goal and outcome

Our goal is the development and product realization of a new system where anyone can easily conduct glycan analysis including the identification of isomers. By making such a system available in society, we hope that glycan analysis will become familiar to many life science researchers who have kept a distance from glycans, and by increasing the

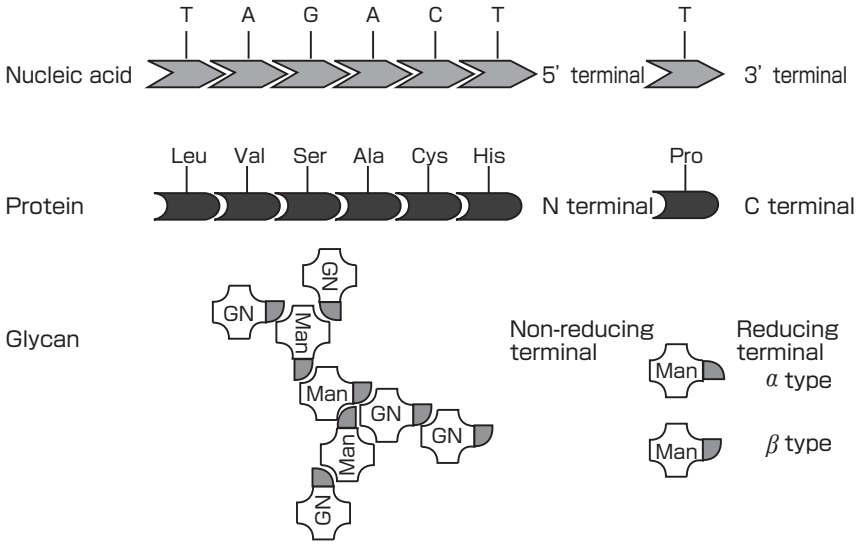

Fig. 1 The reason why it is difficult to conduct sequence analysis of glycan
In glycans, the reducing terminal and non-reducing terminal of sugar bond together. There are four bonding sites (shown as fan-shaped concave parts) in the non-reducing terminal, and there are α and β stereoisomerisms in the bonding sites of the reducing terminal (shown as fan-shaped convex parts). With this configuration, complex isomers with branching structures are formed.

number of people who study glycans, the discoveries of new glycan functions and glycan biomarkers that were formerly unknown are expected to increase. The findings of new glycan functions invite more technological developments, and glycotechnology will spread into the life science research sites. We drew such an outcome.

3 Scenario for the rapid analytical system for glycans

The scenario to achieve the goal did not exist from the beginning. Since the MALDI-QIT-TOF MS was a new type of spectrometer, we did not know what kind of data could be obtained when the glycans were analyzed using this device until we actually did the measurements. First, there was the idea that the laws of fragmentation would be found by analyzing a number of glycans, and then the structure could be estimated from the MS^n [Term 3] spectra based on the laws. We collaborated with the Computational Biology Research Center, AIST, and although we obtained some research results, they did not lead to practical use.[11]-[13]

Under the thinking that identification of isomers is the heart of glycan analysis, we compared the MS^n spectra of multiple glycans with the same molecular mass. It was found that with MS^2 some gave extremely similar spectrum while most isomers gave different spectra when compared up to MS^3.[2] On the other hand, cloning had been done for almost all human glycogenes at that point in the Project, and there was a library of glycosyltranferase[Term 5] coded for each glycogene. Since the glycosyltransferase is extremely specific, it is possible to selectively synthesize a desired isomer by selecting the appropriate enzyme.[14] Therefore, we considered the following scenario (Fig. 3).

First, several types of reference glycan with known structures are purchased, and variations of the reference glycans are increased by extending the sugar chain specifically using glycosyltransferase. Next, the MS^n spectra of each reference glycan are measured and made into a database as fixed values

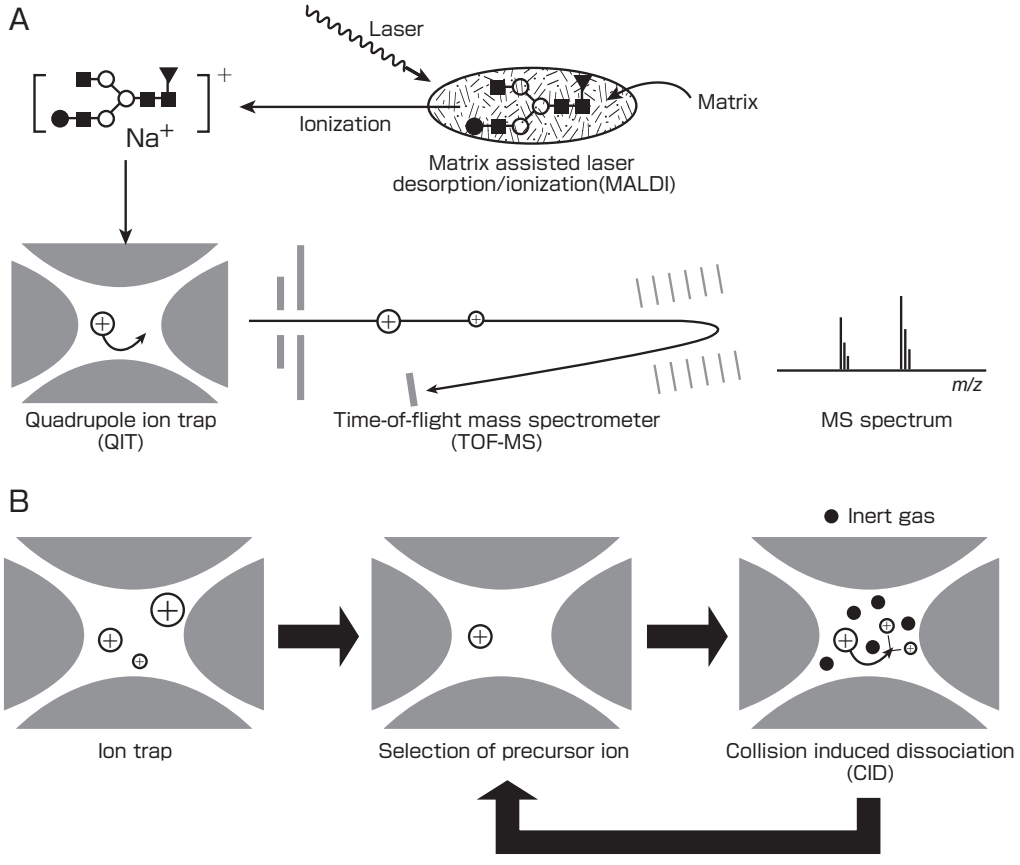

Fig. 2 Schematic diagram of the MALDI-QIT-TOF MS

A: Ions are produced (in the figure, sodium ion adduct is given as an example) when laser is irradiated on the sample that is a mixture of a glycan and a matrix. After the ion is captured in the quadrupole ion trap, it is sent to the time-of-flight mass spectrometer, and the time taken to arrive at the detector is measured. The value where the time is converted to mass-to-charge ratio (*m/z*) is displayed as the MS spectrum.

B: The ion trap captures the ion that is in a certain range of the *m/z* value. When the ion is sent to the TOF-MS, the MS spectrum can be obtained. Also, ions that do not have a certain *m/z* value can be eliminated from the captured ion (selection of precursor ion[Term 4]). When the ion and inert gas collide, the ion dissociates into smaller ions (collision-induced dissociation). The MS^2 spectrum can be obtained by sending the dissociated ion to TOF-MS. If the selection of precursor ion and collision induced dissociation are repeated using the dissociated ion, the MS^3 spectrum is obtained. Theoretically, by repeating the procedure n times, the MS^n spectrum is obtained.

for each glycan. Algorithm to estimate the glycan structure by comparing the MS^n spectra of the analyzed sample and the spectra in the database is developed. Then, the interface software for linking the structure estimation algorithm and the MS operation software is developed. Finally, these are all integrated to create a product, the rapid analytical system for glycans with excellent stability, reproducibility, and ease of use.

4 Development of the elemental technology

To realize the aforementioned scenario, a rapid analytical system for glycans was developed jointly by three parties: AIST, Mitsui Knowledge Industry Co., Ltd., and Shimadzu Corporation. AIST was in charge of the construction of glycan spectra database using the resource from the glycosyltransferase library; Mitsui Knowledge Industry worked on the structure estimation algorithm since it had experience in glycan informatics such as glycogene search; and Shimadzu, the manufacturer of MALDI-QIT-TOF MS, was in charge of the MS and interface software. The details will be explained below.

4.1 Construction of the glycan MS^n spectra database

4.1.1 Selection of the glycan labeling agents

In glycan analysis, generally, fluorescent labeled glycans or permethylated glycans are used, and it is rare to analyze glycans with no derivatization. Therefore, it is necessary to create a database for derivatized reference glycans. In this case, since it was not realistic to prepare several types of derivatives for one glycan, it had to be narrowed down to one. There are several fluorescent labeling agents for glycans, and 2-aminopyridine (PA) is used frequently in Japan, while 2-aminobenzamide (2-AB) is regularly used in Europe and the USA.[15][16] There are personal preferences for fluorescent labels among researchers, and there were people in the NEDO Project who promoted other labeling agents such as pyrene derivatives or 3-aminobenzoicacid (3-AA). As guidance for selecting the labeling agents, there were three points: ionization efficiency in MALDI, information volume of the MS^n spectra obtained by low energy CID,[Term 6] and wide usage in glycan research labs. In other words, focus was placed on sensitive detection, slight structural change that was reflected in the MS^n spectrum, and widespread use by many people. After various deliberations, we decided on PA that was used frequently in Japan because it had good balance in terms of sensitivity and MS^n, and this agent was selected as the glycan labeling agent for the database.

4.1.2 Maintaining the reproducibility of data

To use the database, the reproducibility of data is essential. However, in the tandem MS by low-energy CID, the spectra fluctuated depending on the energy intensity on the precursor ion (CID energy), and since it was impossible to strictly control the energy, it was necessary to devise a way to obtain good reproducible spectra. We focused on the fact that almost the same MS^n spectrum could be obtained every time even if the CID energy fluctuated, if the spectrum was measured at the CID energy when the precursor ion almost disappeared (Fig. 4). Therefore, after various trials, we established a standard of measuring the MS^n spectrum at CID energy at 15 % or less of the maximum peak of precursor ion intensity.[17] Moreover, we ensured that, for each type of precursor ion, the variations in real measurement values could be absorbed by storing two spectra data for MS^2 and three spectra data for high-level spectra of MS^3 or higher where the data tended to fluctuate.

4.1.3 Unification of the measurement mode and the precursor ion species

The mass spectrometer is a device that ionizes the molecules and separates and detects the ion by mass-to-charge ratio. The measurement mode for analyzing positively charged ion is called the positive ion mode, and the mode for analyzing negatively charged ion is called the negative ion mode. When constructing the database, we considered which measurement mode should be selected. While we were attracted to the negative ion mode since there were reports of production of special fragments that might be useful in the glycan structure estimation,[18][19] we selected the positive ion mode since the negative mode was disadvantageous in terms of ionization. In

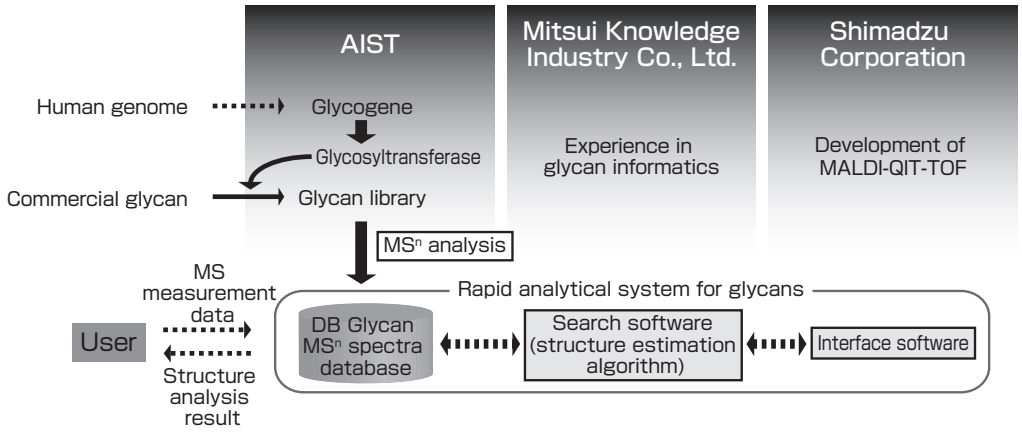

Fig. 3 Elemental technologies, their background, and mutual relationships

ionization, various ions with added protons, sodium ions, and potassium ions could be produced, and there were differences in fragmentation according to the adduct ion. Therefore, it was necessary to determine the ion species. In case of glycans, it was found that the spectra difference readily occurred between the isomers by using the sodium ion adduct as the precursor rather than using the proton adduct as the precursor, and we selected the sodium ion adduct. Also, there were reports that for the proton adduct of fucose containing glycans, fucose rearranged in the ion trap.[20] and the sodium adduct was considered more appropriate.

4.1.4 Selection of the matrix

In MALDI, the selection of the matrix is important. Glycans degrade readily in acid conditions, and it is common that decomposed matters may be generated during ionization if the acid matrix is used. Also, due to the unevenness of sample concentration that may occur when creating the co-crystals of the sample and the matrix, there are problems of so-called "sweet spots" or the area from which the signals can be obtained is limited when irradiating with lasers. Since the data reproducibility is necessary in the database, it is desirable that the measurement be done automatically with no inclusion of operator bias, but sweet spots make that difficult. We considered various matrices, and selected a method of creating homogenous microcrystals with no sweet spots by making the co-crystals and then re-crystalizing by ethanol, using 2,5-dihydroxy benzoic acid (DHB) as the matrix.[21] The DHB was an acid matrix and glycans degraded during ionization in some cases, but this method was best considering the issues of sensitivity and sweet spots.

4.1.5 Glycan preparations for constructing the database

Since there were only a few types of pyridylaminated glycans that were commercially available, we modified the commercial pyridylaminated glycans using the glycosyltransferase at the Research Center for Medical Glycoscience to increase the variation. In the synthesis for the glycan library, Researcher Hiromi Ito of the RCMG (currently at Fukushima Medical University) played a central role. The above measurements were conducted using the samples, and ultimately 2,897 spectra were incorporated into the glycan MS^n spectra database.

4.2 Structure estimation algorithm and search software

4.2.1 Glycan description language

The database stores the structural information of glycans and the MS^n spectra data for those glycans. For the glycan structure, it is necessary to describe the branches and bonds, and these were difficult to handle with the computer. There was no standard method for data description at the beginning of the project. Therefore, we developed the carbohydrate sequence markup language (CabosML) using XML.[Term 7][22] The structure described in the CabosML format was stored in the database, and various applications were developed using the CabosML format for the input data.

4.2.2 Elimination of the noise peaks

With the search software, the structure estimation is done based on the similarity of spectrum form including the peak intensities, by comparing the measured MS spectra of unknown structures and the spectra of known structures stored in the database. If the noise peak is included in the reference spectra data, the search precision decreased due

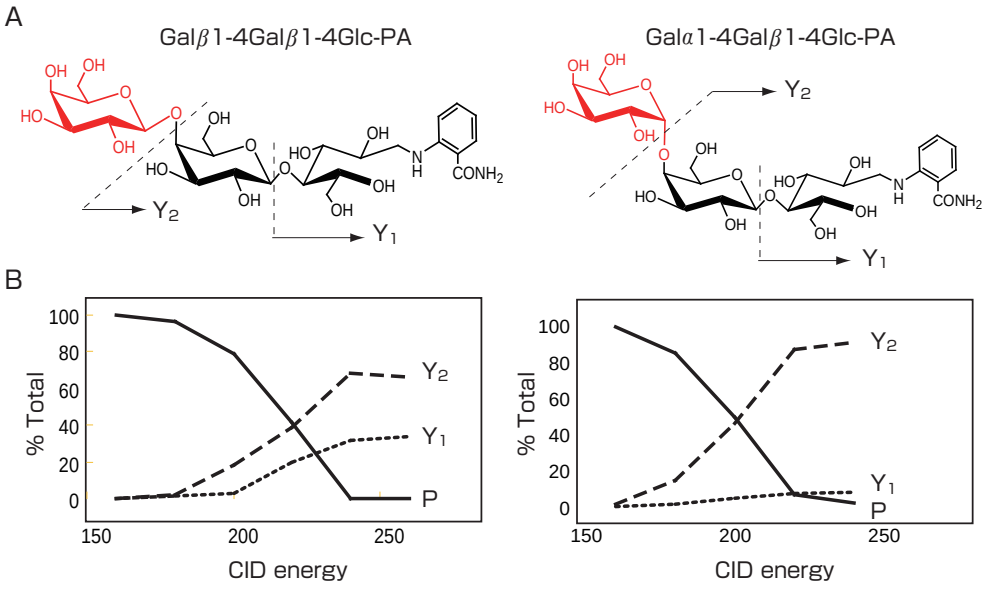

Fig. 4 Fragmentation of the glycans by ion trap

The glycans on right and left are isomers in which only the configurations of terminal galactose (shown in red) are different. The graphs beneath them show the changes in peak intensity of the fragment ion produced from each glycan when the CID energy is increased. In the CID energy where P almost completely disappears (around P < 15 %), the ratio of Y_1 and Y_2 become almost constant. The Y_1/Y_2 ratio that becomes constant is different between the two isomers. P: precursor ion, Y_1, Y_2: fragment ion.

to the noise peak. Therefore, the theoretical fragments were calculated from the glycan structure, and only the peaks that matched the monoisotopic mass[Term 8] of each fragment were extracted from the spectra data. The peak list including the intensity information was set as the spectra data for search.

4.2.3 Detection of the glycan peaks

The MS spectra of the samples include the peaks of foreign materials, decomposed matters, as well as peaks deriving from the matrix, other than the peaks of the glycans that one wishes to study. Therefore, the first step of database retrieval is to detect the glycan peak from the measured spectrum. While there are various methods, in the structure estimation using the database, there is no meaning in analyzing the glycan peak not registered in the database. Therefore, judgment for glycan peak detection was made according to whether the glycan peak was registered or not registered in the database (Fig. 5).

The interface software that will be described later delivers the sample information such as the labeling agent and the adduct[Term 9] along with the peak list. The search software reviews such information, searches for the precursor ion with the same *m/z* value as the registered peak, and notifies the interface software of the found peak as being a glycan-derived peak. If it is not found, the glycan peak is detected in the Extended Search mode described later (see Section 4.2.7).

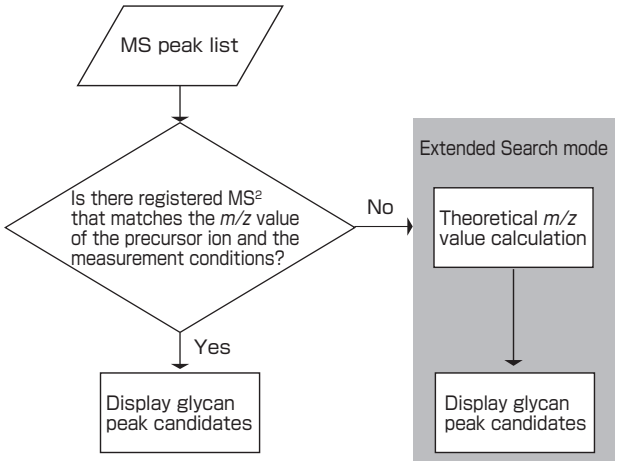

Fig. 5 Logic flow of the glycan peak detection

4.2.4 MS^2 search

In the MS^2 search, the similarities between the MS^2 spectra of the sample and the MS^2 spectra in the database are referenced. The similarity is calculated using the vector composed of the *m/z* value and the peak intensity of each peak, and the glycan with the value at the threshold or higher will be given as the candidate for the estimated structure.

4.2.5 MS^3 search and rapid identification

When there are multiple candidates for the estimated structure, narrowing down is done by measuring MS^3. In MS^3, each peak on the MS^2 spectra will be the precursor ion candidate (Fig. 6). Too much time and labor will be spent if the MS^3 is measured for each peak of the MS^2 spectra and then compared with the spectra in the database. Therefore, "which peak should be measured to narrow down to one candidate" is projected using the MS^3 spectra in the database, and rapid identification is achieved by sending this information to the interface software.

4.2.6 Extended Search mode

As mentioned earlier, we unified the fluorescent labeling agent for glycans to PA for the database construction. Therefore, glycan structure estimation cannot be done if the user is using a different labeling agent. This will be a major weak point when introducing this database to Europe and the USA where other labeling agents are used. Therefore, we solved this problem by devising a search method, without

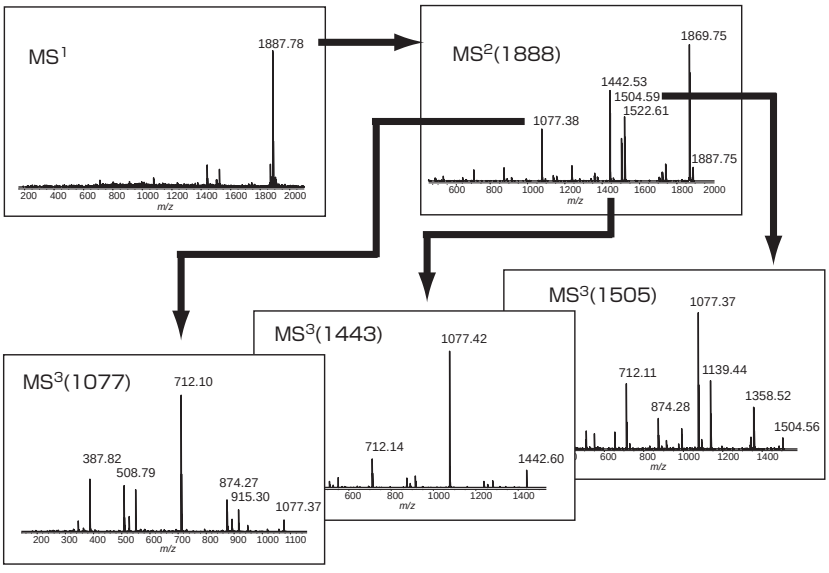

Fig. 6 Tree structure of the multistage MS

adding to the content of the database. This is the method called the Extended Search mode. In the Extended Search mode, the following search is conducted.

4.2.7 Detection of the glycan peak in the Extended Search mode

The user enters the sample information such as the labeling agents and adduct of the glycan to be analyzed. The system calculates the *m/z* values of various glycans that can be conceived theoretically by combining the glycans based on the given information, and creates the aggregation of these *m/z* values. If the *m/z* value of the peak of MS spectrum measured exists within the aforementioned *m/z* value aggregation, that *m/z* value is shown as the glycan peak candidate (Fig. 5 right).

4.2.8 Presentation of the key fragment

Since the labeling agent for MS^2 spectrum of the presented glycan peak candidate is different, the similarity with the spectra in the database cannot be determined. However, there are many fragments from which the labels have fallen off that appear in the MS^2 spectra. By comparing the MS^3 spectra assuming these are precursor ions, it seems to be possible to estimate the structure. What should be noted here is that each peak of MS^2 spectra may not necessarily be composed of a single fragment. Most peaks are composed of a combination of multiple fragments with the same *m/z* value. Comparing this with the MS^3 that sets such a peak as precursor ion is not useful in structure estimation. To conduct structure estimation, the peak composed of a single fragment structure without the labeling agent (key fragment) must be selected as the precursor ion of MS^3. In the N-glycan, the chitobiose section of the reducing terminal is readily cleaved, and the *m/z* value of the fragment cleaved at this section theoretically cannot contain other fragment structures, and therefore it can be used as the key fragment (Fig. 7).

In the search software, the *m/z* value of the key fragment is calculated from the *m/z* value of the precursor ion of the glycan peak candidate, that is, [*m/z* value - (labeled GlcNAc)] or [*m/z* value - (Fuc labeled GlcNAc)], and search is done to see whether this exists in the MS^2 data measured by the user. However, in the structure where the Fuc is bonded to the reducing terminal GlcNAc (Fig. 7A), the peak [*m/z* value - (labeled GlcNAc)] is not formed, and only [*m/z* value - (Fuc labeled GlcNAc)] is given (Fig. 7B, corresponding to the key fragment). Considering these factors, the search is conducted according to the logic flow in Fig. 8.

4.2.9 MS^3 search

When the MS^3 measurement data of a key fragment is sent to the search system, the search is conducted of the MS^3 data in the database to look for items with high similarity. In a case where there are several glycan structure candidates with a degree of similarity over the threshold, the *m/z* value of the precursor ion of MS^4 data that can be used to narrow down the candidates is sent to the interface software to conduct the measurement and search. If the MS^4 candidate does not exist in the database, the search is terminated.

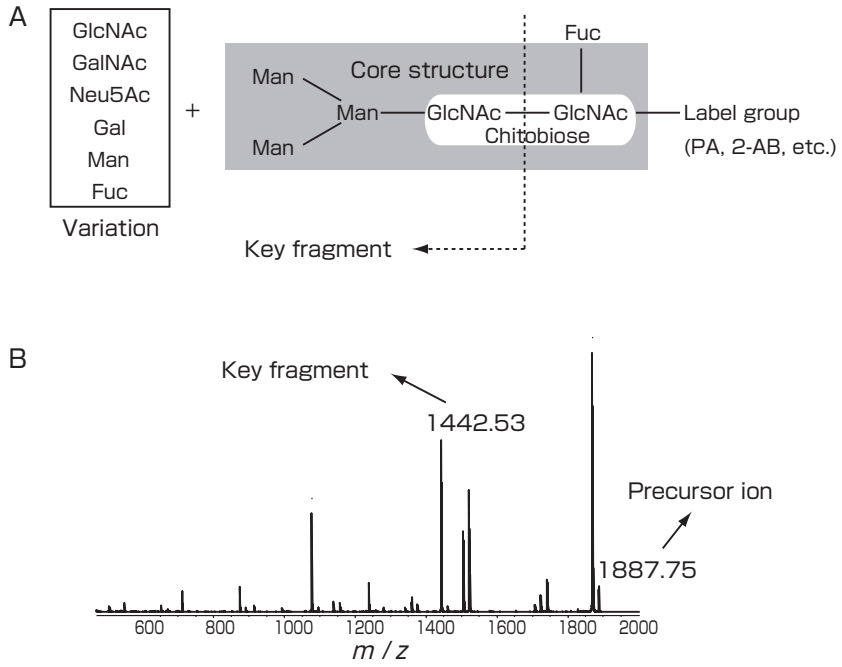

Fig. 7 Structural characteristic of the N-glycan and key fragment

A: For N-glycans, variations occur when various types of sugar in the white area join by various bonding patterns to the Man on the left side of the core structure.

B: In the MS^2 spectrum of N-glycans, the key fragment forms a large peak. This peak is formed by single structure that does not contain the label.

4.3 Interface software

The rapid analytical system for glycans aims at anyone being able to reach the estimated structure of the analyzed samples by measuring the MS spectrum through the navigation by the search software. To realize this, an interface software that can be used "easily by anyone" is necessary, as well as linking the analysis software that controls and operates the mass spectrometer, the search software, and the database. In the interface software we developed, we implemented the function to deliver the data from the search software to the analysis software with a click of a button, as well as the function to deliver the spectra data from the analysis software to the interface software with one click. The outline of the analysis flow using the interface software is shown in Fig. 9.

4.3.1 Measurement aid through the interface software

The measured MS spectrum data is delivered to the interface software after being converted to the peak list format composed of the *m/z* value and peak intensity value of each peak by the analysis software.

In the interface software, the search parameters are entered first. The parameters include the labeling agent, sample information such as adducts, as well as the tolerance setting and others. Next, the parameters set by the user are added to the peak list information obtained from the analyzing software, and are sent to the search software. The interface software receives the list of precursor ions that should be measured next from the search software and displays them. Items are prioritized in the lists, and the precursor ion information selected by the users are sent to the analysis software.

The display method of precursor ions that are measurement candidates was selected based on the visual ease of understanding for the user. Specifically, by organizing the measurement candidate in a tree format by each search ID, the

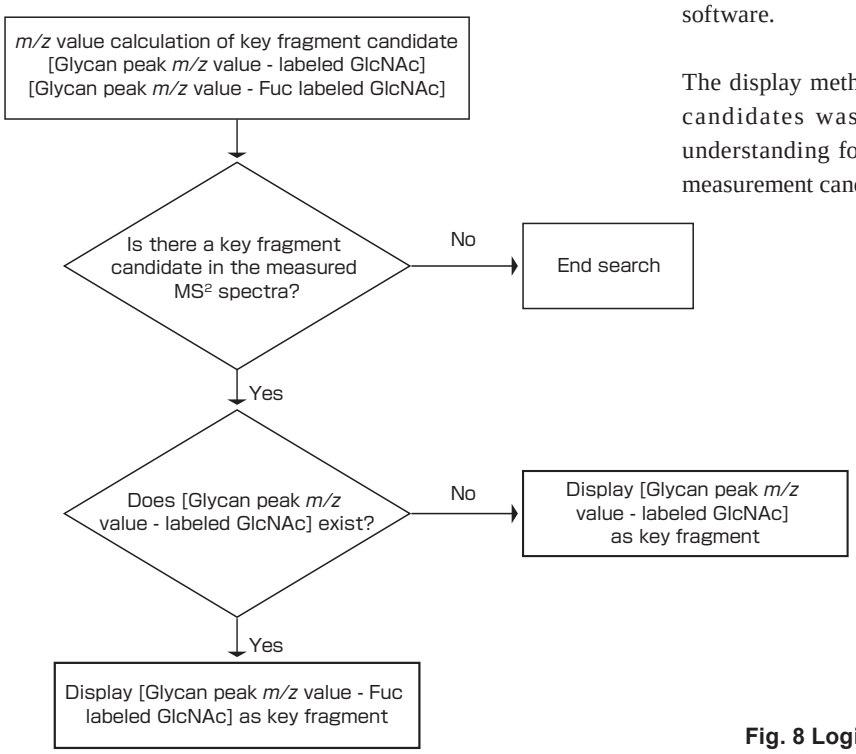

Fig. 8 Logic flow of the key fragment identification

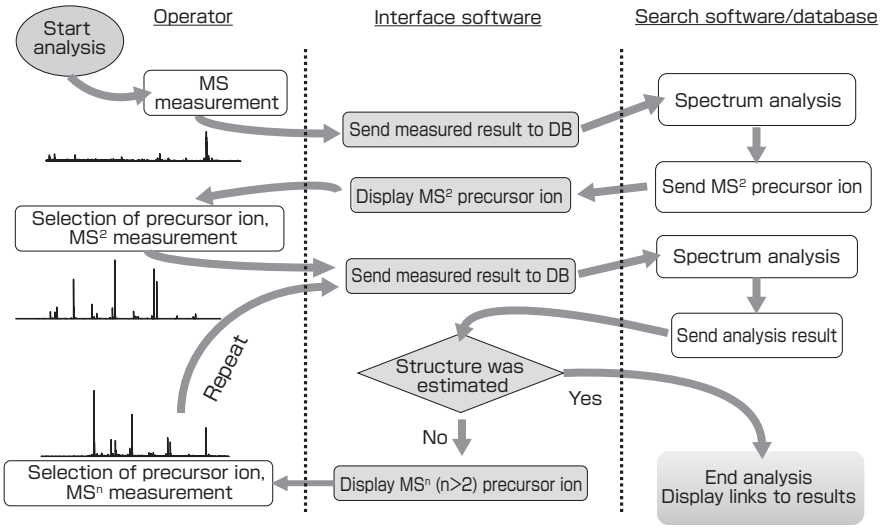

Fig. 9 Analysis flow of the rapid analytical system for glycans

The interface software executes the functions shown in the center, and mediates between the operator and the search software.

multiplier of the MS^n currently measured and the *m/z* values of the precursor ion in the previous step can be understood readily. Also, the icons of each precursor ion have different colors according to the acquisition status of the spectra data (Fig. 10).

In the analysis software, the precursor ion information received is reflected in the setting section of the MS^n analysis condition. The user sets the appropriate CID energy value to execute the MS^n analysis. The MS^n spectrum data obtained is sent again to the interface software to conduct the search.

4.3.2 Displaying the results

The interface software is implemented with the function to display the message from the server. If the structure estimation result is obtained by the search software, a message is displayed and the user discerns the end of the analysis. The structure estimation result is aggregated on the HTML format web page through the search software, and viewing is done on the Internet browser. The interface software receives notification of the web address of the estimation result page from the search software, and with a button click, the browser opens to display the web page for the structure estimation result. The estimation result shows the score in graph format, the estimated glycan structure, and the link to the Japan Consortium for Glycobiology and Glycotechnology Database (JCGGDB) for viewing the information for the related glycan structures (Fig. 11).

5 Intellectual property strategy

For product realization, securing of the intellectual property rights is essential. The patent for this system was filed under the title “Method of identifying sugar chain structure and apparatus for analyzing the same.”[23] This is the patent to claim the method and the apparatus that embodies the method. The method of estimating the structure by matching the spectrum of the analyzed substance to the spectra in the database is not particularly novel, and a prior patent had been already filed, even limited to glycans. After consulting the patent attorney, we decided to emphasize the idea that would be the core of the system. This was based on the idea that “the heart of glycan structure analysis is the identification of isomers,” and it was a method for searching the MS^3 spectrum that is expected to show the most difference in spectra among the isomers in the database when estimating the structure by matching the high-level tandem MS spectrum of MS^3 or higher, and comparing only the MS^3 spectrum with a degree of similarity at a certain value or less. In the actual patent claim, this idea was expanded to MS^n, not just MS^3. Care had to be taken because the feeling of the researcher was that the patent could be obtained just for the method that used the glycan MS^n database that never existed before. This patent was registered in Japan, Germany, and China after a few office actions.[24][25]

For the intellectual property strategy, there is the method of obtaining the rights by claiming the copyright of publication, other than obtaining the patent. The rapid analytical system for glycans is composed of the mass spectrometer, the glycan MS^n spectra database, the structure estimation algorithm, and the interface software. The glycan MS^n database was one of the intellectual foundations, and since it could be used independently, it was created and registered as intellectual property as a unique publication of AIST.[26] The search software with the structure estimation algorithm was registered as a joint publication of Mitsui Knowledge

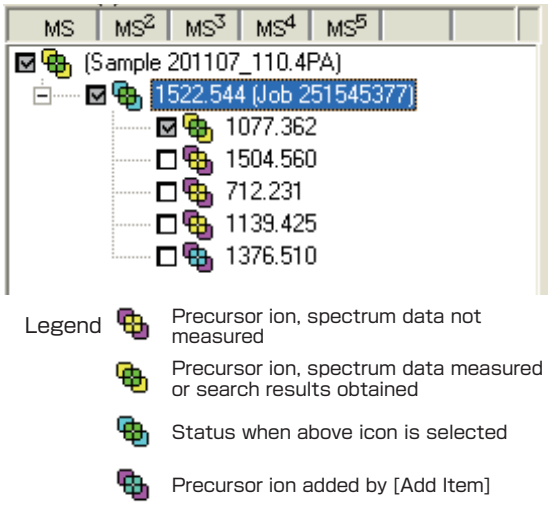

Fig. 10 Display of the precursor ion candidates

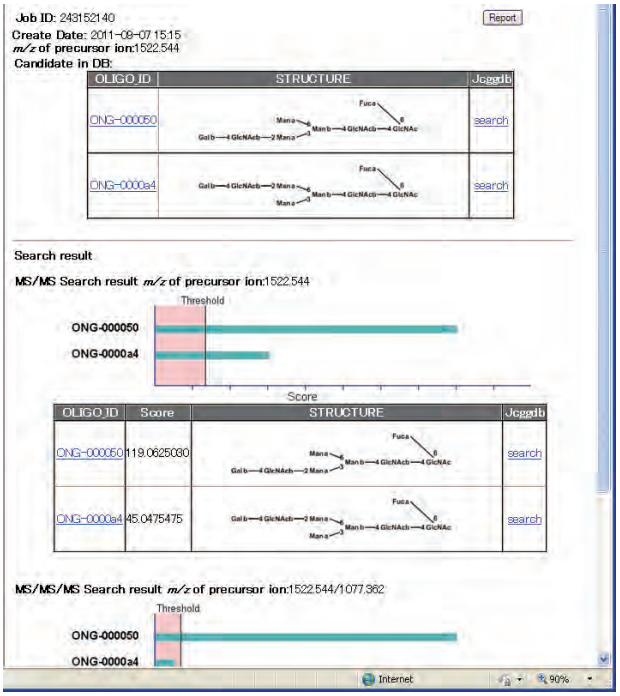

Fig. 11 Screen of the search result

This shows matching glycans where the score bars are within the threshold (red area). When one clicks on [Search] in the Jcggdb section, the page with the information for that glycan opens.

Industry Co., Ltd. and AIST under the title "CASIS."[27] The mass spectrometer and the interface software were already covered by the patent and copyright of Shimadzu Corporation.

Full Research toward product realization after filing the patent was continued in the framework of patent realization joint research that was handled by the Intellectual Property Division of AIST at the time. Product realization might not have happened without this framework.

6 Product realization of the rapid analytical system for glycans

To promote diffusion of this system, Shimadzu Corporation worked on the development of the product as a package of three components (database, search software, and interface software). The MS^n spectra database for glycans in this system was created for the glycans that are synthesized in human cells. Therefore, the focus was set on fields that studied humans, and the antibody pharmaceuticals for which the development was increasing were set as the main target at the time. Joint research for patent realization was started among three parties, AIST, Mitsui Knowledge Industry Co., Ltd., and Shimadzu Corporation.

In the SG Project, since the data collection and investigation were done on one mass spectrometer, in product realization, it was necessary to check whether correct search was conducted after considering the device differences among the spectrometers. Therefore, several devices that were the same as the spectrometer used in the Project were obtained, the data measured by these devices were compared, adjustments in the search algorithm were made to avoid large effect on search due to the data differences among devices, and the method for inspecting the device condition was established. Concurrently, we attempted the expansion of the glycan MS^n spectra database. There was a plan to add more glycan MS^n spectra to the database, mainly for the N-bond glycans containing sialic acid. However, this data collection was stopped because the antibody pharmaceuticals obtained from the Chinese hamster ovaries (CHO) cells, which is the production host necessary for the biopharmaceuticals, contained hardly any sialic acid and because the measurement of glycans containing sialic acid required a separate chemical treatment called methyl esterification. It was decided that the effort should be spent on completing the software. The early commercial version of the Accurate Glycan Analyzer, the rapid analytical system with functions developed during the Project, was released (June 2010).

At the time, this system was capable of estimating the structure of the PA-labeled N-glycans and the sugar alcohol of O-glycans in which reducing terminals were reduced. Although it caught attention of various companies and researchers, there were requests for improvements from people who used labeling methods other than the PA label. Therefore, we planned the development of an improved system that could meet the user demands, such as the function to enable structure estimation for glycans treated with 2-AB or 2-aminobenzoic acid (2-AA) or glycans labeled with various agents other than PA (see aforementioned Extended Search mode), the function where the users themselves can expand the database, and the data verification function that allows search using the data registered by the user.

Considering the required specifications to achieve all of the above functions, it was found that the development budget went beyond the joint research budget, and the narrowing down of the core functions became necessary. Upon discussion among the three parties, we reached the conclusion that priority should be given to incorporating various labels. We decided to develop the improved rapid analytical system for glycans with the Extended Search mode, where the glycan structure estimation could be done using the existing database in the case where user's labeling agent is used, as well as the commonly used glycan labels such as PA, 2-AB, and 2-AA. We were able to release the Accurate Glycan Analyzer 2, the improved rapid analytical system for glycans that can respond to various glycan labeling agents (December 2011).

7 Results and their significance

7.1 Continue on without being swayed by the trend

The first version of the rapid analytical system for glycans was released in June 2010, and the second version that was improved based on user demands was released in December 2011. In retrospect, none of the competitors at the time when we started the development achieved product realization. To get the product released, one must continue on steadily toward product realization without being swayed by the prevailing trend. Pertaining to this Project, in the beginning, the development of a new glycan analysis method using mass spectrometry was the central issue of glycomics, and active research was being done throughout the world. Yet, in about three years, the interest shifted to disease biomarkers. Many people who had been studying the glycan analysis method jumped to the glycan analysis of various clinical samples, and competed in publishing papers and filing patents for biomarkers one after another. The development of the analysis method was incomplete, but they threw that out and shifted to the next trend. Biomarker search became the theme in NEDO's glycan projects, and the authors were also swept up in the wave and had to give up system development under the NEDO Project. Fortunately, research on the rapid analytical system was continued on a separate budget of the Intellectual Property Division from 2007, and finally we achieved the above results. Currently, this system is employed

at 10 universities, companies, and research institutions in six countries (as of April 2015). Also, the actually measured MS^n spectra database was released as an open database. The open version does not involve numerical data but consists of images of MS^n spectra, and this maintains academic usefulness while avoiding the pirating of the rapid analytical system for glycans.

7.2 Power of software backed by experimental science

The product we developed is the world's first glycan analysis system with an actually measured MS^n spectra database. Anyone can easily identify isomers that are the heart of glycan analysis. Currently, there is no other glycan analytical system using an actually measured database other than our product on market. This system was born by combining hardware consisting of a new type of mass spectrometer and three software (spectra database, structure estimation algorithm, and interface software). To maximize the capacity of the hardware, the power of software that captures the on-site demand is necessary. Just as the car navigation system can guide a person to a destination even in an unknown place, this system interactively leads the user who may not know anything about glycan analysis to the estimated structure. However, the development of this system was not done just by information science, and we would like to emphasize that the foundation is the experimental science of glycogenes and glycan analysis.

It should be noted that glycan analysis software using a theoretical database is available on the market, but there is none that is of world standard at this point, including the free products.

7.3 Were we too ahead of our time?

The heart of glycan analysis is how to identify the isomers. We developed this system for that purpose. While it is very clear that the differences in glycans by positional isomerisms or stereoisomerisms lead to different functions in the body, people who study this are the glycobiology researchers who have been studying glycans from the beginning. For ordinary life science researchers, knowing the molecular mass of a glycan is sufficient, and they do not even imagine that there are possibilities in isomers. While this situation may change as the functional clarification of glycans progress, we feel that we were ahead of our time in developing the rapid analytical system for glycans that allows anyone to easily study glycans.

8 Toward the realization of the outcome

The real outcome is the situation where the knowledge of "glycans," which is the third chain of life, is used effectively and routinely in life innovation such as drug discovery and regenerative medicine. In other words, it is a situation where studying glycans is a standard procedure. The lectin microarray and the rapid analytical system are only parts of this goal. In fact, the technological foundation of glycoscience is still fragile. The glycans for which we created the database is limited to human glycans. Mucin, which is the viscous component of the mucus and bodily fluids, is a giant glycoprotein and is said to be related to disease, but the understanding of its function has not progressed because it is difficult to analyze. Podocalyxin that was recently reported as one of the iPS markers is also a mucin-like protein.[28] Currently, research is underway for the analysis of O-glycans that is a mucin glycan. For glycan analysis, it is necessary to pretreat the glycoprotein and derivatize it to a state where its glycan be analyzed, but this pretreatment process is a barrier in the diffusion of glycan research and must be improved (Fig. 12). These issues are only examples, and we face huge obstacles when the issues of "synthesis" and "function" are combined.

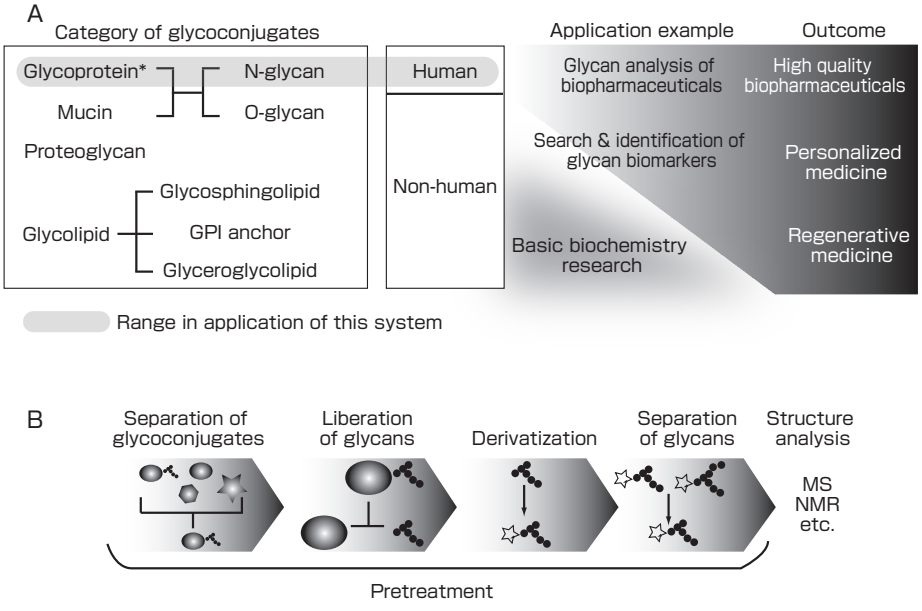

Fig. 12 Positioning of this system in the glycoconjugate research and future issues

A: The glycans exist as glycoconjugates bonded to proteins and lipids. Although there are various glycoconjugates, this system targets only human N-glycans of glycoproteins. ∗Mucin and proteoglycan are also glycoproteins, and therefore, to differentiate from those, here, we refer to glycoproteins with 50 % or less sugar content.

B: Multistage pretreatment is necessary for analyzing the glycoconjugates. This system supports only the final stage or the structural analysis.

Although industrialization is necessary to realize the outcome, and it has been said that glycan research is Japan's stronghold, in the current situation, there are very few cases where this research was released to society as industrial science. In this sense, in the future, AIST that has accumulated various glycan resources (knowledge, technology, and personnel) must not just deepen and develop the technology but must send this industrial technology into society through collaboration with companies. On the other hand, the important requirement for a company to send a product into society is profitability. When one attempts to realize new glycan technology as products, this is the first problem one must face. To expand the market, it is necessary to have the pharmaceutical companies and life science researchers to widely recognize the importance of glycans. To do so, it is necessary to continue to elucidate glycan functions and accumulate case studies with impact. Although this may be a commonplace conclusion, in order to realize the outcome, we believe we must steadily engage in a wide-range of research from basic glycan research to transferring the technology to the companies.

Terminologies

Term 1. High-energy CID: Here, this term refers to the collision-induced dissociation in magnetic sector MS and time-of-flight MS. The molecule is characterized by the fact that although it may be cleaved in various places due to collision, multiple places are not cleaved at the same time.

Term 2. Omics: Research method in which the total biomolecular species in an organism are comprehensively researched. If the subject is genes, it is genomics. If it is protein, it is proteomics. In the beginning of the 2000s, various omics were born including metabolomics and glycomics due to the rapid development of mass spectrometer.

Term 3. MS^n: Multistage tandem MS. See Fig. 2B for MS^n using the ion trap.

Term 4. Precursor ion: Ion selected to be fragmented in the tandem MS.

Term 5. Glycosyltransferase: The enzyme that transfers sugar and extends the sugar chain. It has extremely high specificity for bonding position and stereoisomerism. Therefore, precise synthesis of glycans is possible by selecting the appropriate glycosyltransferase.

Term 6. Low-energy CID: Here, this term refers to the collision-induced dissociation in the quadrupole MS and ion trap MS. The weak bond in the molecule is cleaved. In glycans, fragments where several places are broken are often observed. If a charged glycan labeling agent is used, the ionization efficiency may increase, but only the fragments containing the label are observed and the information volume decreases. Even with a non-charged labeling agent, in the case where there is a large difference between the observation of fragments with and without the labeling agent, the spectra information decreases.

Term 7. XML: Acronym for extensible markup language. A type of computer language to structuralize and describe the text by designating the logical structure and meaning of the text with tags called the markups. Data sharing and processing by program is facilitated by structuralizing and describing the text.

Term 8. Monoisotopic mass: Elements have various natural isotopes. The mass calculated using only the mass of the maximum isotope abundance ratio for each component element of a molecule is the monoisotopic mass.

Term 9. Adduct: Here, this term refers to the additional ions that are generated during the ionization of glycans in MS. There are proton adducts, sodium ion adducts, and others.

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Discussions with Reviewers

1 Overall

Comment (Tai Kubo, AIST)

Glycomics, or systematic approaches to glycobiology, started far behind proteomics and genomics. It was mainly due to the complexity and diversity of the structure and functions of glycans. However, national projects activated and boosted the glycan research in Japan, and unleashed innovation toward biotechnologies and drug discovery as well. We are now advanced in the field and are leading the world. In the advancement, the researchers at AIST played essential roles and contributed a lot. The authors of this article were in charge of the analysis of glycan structure in the "Structural Glycomics (SG) Project". This paper describes the processes how the authors tackled the encountered problems when they applied mass spectrometry (MS) to analyze the glycan structure, and how they accumulated the knowledge to establish an integrated glycan analysis system with a database. They also created the system with the intellectual property aspect in mind, and it is now open to the public with high utility value. It can be said that this paper is written along the objective of *Synthesiology.*

Comment (Hiroaki Tao, AIST)

This paper is a description of the background, elemental technologies, development scenario, product capability at this point, and future prospect in realizing the outcome, or the product realization of the "glycan analytical system that can analyze the isomers and branch structures." This is necessary for future glycan research and glycotechnology. The specialties of three parties including AIST, an analysis device manufacturer, and an information processing company, as well the strategy to fuse their specialties are aptly described. It contains useful information for researchers and engineers who aim to develop analyzing devices and analytical systems that are necessary in pioneering new academic fields, and I feel the paper is valuable for publication in *Synthesiology.*

2 Research scenario

Comment (Tai Kubo)

The scenario in Chapter 3 is an important point for *Synthesiology.* Please create a schematic diagram that shows the flow from R&D to product realization, including the development of individual elemental technologies described in Chapter 4.

Comment (Hiroaki Tao)

The scenario in Chapter 3 should be described as "3 Scenario for the development of a rapid analytical system for glycans." I think the overall strategy can be understood more readily if you show in a diagram the technologies that the companies already possessed, for example, the human glycan gene cloning and the glycosyltransferase library of AIST and the MALDI-MS of Shimadzu, and the technologies that were newly developed for this system.

Answer (Akihiko Kameyama)

I added "Figure 3. Elemental technologies, their background, and mutual relationships" in Chapter 3.

3 Elemental technology

Comment (Tai Kubo)

In Chapter 4 you describe the processes to solve various problems that you encountered during the development of the glycan structure analysis system, and we can imagine that enormous effort was spent during the process. However, sometimes the explanations are too detailed with specialized terms. Please review the expressions overall with the readers outside of this field in mind. I would suggest that additions of a terminology section and a schematic diagram of the MS method might be helpful for a broad range of readers.

Answer (Akihiko Kameyama)

I added a schematic diagram of the mass spectrometer used in this research to Chapter 2 along with the description. I also added a list of terminology at the end of the paper.

4 Search software and analysis software

Question & Comment (Tai Kubo)

You describe your efforts as you grope toward a solution one by one: for example the reproducible measurement of glycan MS, the structural description language to create a database, the method to extract the glycan peaks in the MS, and so on. All these developments must have been done with universal applications in mind. What is the world standard for search and analysis software? Is there compatibility with databases other than JCGGDB? Please address the comparison and compatibility with other competitors.

Answer (Shuuichi Nakaya)

I don't think there is a world standard for glycan search or analysis software. The commonly used search software is the Glyco Mod Tool that is registered in the ExPASy protein server. It is a tool on the web that can be used free of charge. However, this is not for structural analysis, but it detects the glycan peak in the MS spectra and displays the glycan composition that is estimated from the molecular mass. Recently, there is a payware called SimGlycan (Premier Biosoft) that is becoming popular. This is not a database of actually measured spectra, but is a database of calculated fragments. Other than these, software programs that use mass spectrometry are developed and sold individually by each MS manufacturer. For the search using the interface software, currently, we have no compatibility other than with JCGGDB.

Answer (Akihiko Kameyama)

I think the heart of glycan structure analysis is the identification of isomers. In this case, the database of calculated fragments are useless. I have expressed this point in Subchapter 2.1.

5 Results and their significance

Comment (Hiroaki Tao)

In Subchapter 7.3 "Were we too ahead of our time?," I felt that what you really wanted to say or should do were to describe "the

difficulty encountered by people who pioneer in the forefront, and the will to contribute to glycan research no matter how difficult." Therefore, I think you should describe the impact of introducing this system or an example of effective application.

Answer (Shuuichi Nakaya)

This system has been introduced to overseas drug development companies for the analysis of glycan structural differences in the development of biosimilars.

Answer (Akihiko Kameyama)

I believe the impact of introducing this system and examples of effective application will emerge soon.

6 For the realization of the outcome

Comment (Hiroaki Tao)

In Chapter 8, you describe the research targets to which this system can be applied. I think the readers will be able to readily grasp the overall image of glycan research if you show a diagram that explains this systematically. To do this, I think you should show, for example, the overall image of glycans that represent the glycan types, the glycans to which the system can be applied, and the direction of future R&D for the glycans to which the system cannot be applied.

Answer (Akihiko Kameyama)

I added Fig. 12 "Positioning of this system in the glycoconjugate research and future issues" in Chapter 8.

Health care application of gas sensors

— Medical devices of breath analysis —

Woosuck SHIN*, Toshio ITOH and Noriya IZU

[Translation from *Synthesiology,* Vol.8, No.4, p.214-222 (2015)]

For the research goal of exhaled breath detection system in human health care or medical application, gas-selective and sensitive gas sensors have been developed. High performance sensors satisfying the boundary conditions, such as fast response and highly sensitive and selective detection, were developed with three essential components of a novel working principle, nanoparticle technology, and a ceramic integration process of sensing materials.

Keywords : Halitosis sensor, hydrogen sensor, VOC sensor, breath analysis, nanoparticle

1 Introduction

As aging of the population progresses in Japan, preparing sufficient supply of health care devices and services as well as curbing the expense of social welfare are raised as social issues. Against this background, medical examination using the breath has the advantages of being non-invasive, allows easy collection of samples, and provides quick results, and is gaining attention as new diagnostic technology.

The main gas composition of human breath consists of nitrogen that is the most common in atmosphere, carbon dioxide produced by respiration, oxygen that was not consumed, and water vapor generated from bodily fluids. It is composed of more than 100 types of gas components, and the components and concentration offer information that may be useful to monitor health conditions such as the presence of disease or stress. For such analysis or health checkup, technologies are necessary to selectively detect various gas species in the breath, and to measure the concentration of important gas species that are correlated with halitosis, metabolism, and disease.

Private companies already have high levels of gas sensor technology and solid manufacturing technology. Therefore, to differentiate from such technologies, we developed a sensor with a detection mechanism different from the conventional gas sensors. We developed a sensor that rapidly and sensitively measures the halitosis components in the mouth using oxide nanoparticles, and succeeded in commercializing a halitosis detector. Also, we are developing a detector for monitoring hydrogen and carbon monoxide in the breath using a new thermoelectric sensor. Moreover, we are developing a sensing technology utilizing the separation technology of gas chromatography (GC) and semiconductor gas sensors, to detect volatile organic compound (VOC) gas that is thought to be a marker for lung cancer. Currently, we are actively collaborating with medical fields from the perspective of public acceptance, in realizing the practical use of the sensors and systems that we developed.[1]

In the future, the gas analyzer developed in this research will make daily breath monitoring possible and enable self-management of health conditions by individuals, and this will greatly contribute to lowering medical costs. The demand for health care and medical care around the world is projected to increase from 32 trillion yen in 2011 to 40 trillion yen in 2020.[2] Many devices used in health care are imported, and health care business development is difficult. The problems of medical devices are the following: the market is small; collaboration of medicine and engineering, such as, chemical tests for practical use including obtaining official approval is not actively done compared to Europe and the USA; and the Japanese manufacturers have very little experience in development. Therefore, marketing of new technology that can be used clinically in Japan and overseas is difficult through conventional product development. In this paper, we discuss how the R&D achievement of our sensor technology development can lead to the creation of a new medical application, focusing mainly on the health care field.

2 Social demand for the sensors (performance)

Recently, the capacity to monitor health conditions and to predict disease non-invasively and easily without violating the Medical Practitioner's Act is coming into the spotlight.

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One of the simple ways is to make predictions based on biogas, just as in the old days when the physicians made decisions by smelling the odor from patients. Figure 1 is a summary of the sensing methods and the relationships between the physical conditions or diseases and various biogases. A representative example is alcohol detection in the breath, and alcohol breath checkers are commercially available. However, drunkenness is caused by an external factor, and it is not an index of a human state. While, there are devices of urea breath test to find *Helicobacter pylori*, and devices used by asthma patients to measure nitric oxide.

Recently, hydrogen is in focus. Health care supplement business is spreading based on the claim that drinking hydrogen water will make a person vigorous. This keen interest in hydrogen was initiated by a paper published by the Nippon Medical School reporting that cytotoxic oxygen radical, which is considered harmful in high concentration, is reduced by hydrogen.[3] Humans do not produce hydrogen, but hydrogen is produced by bacteria in the intestinal environment. When food is not absorbed in the small intestine, it goes directly into the large intestine where the bacteria grow in large amounts and produce abundant gas.[4]

This means that hydrogen may be produced or not produced in abundance depending on whether the food is agreeable with one's stomach (digestive system). There is a demand for measuring hydrogen, and a hydrogen detector is being developed for this purpose. We are working on monitoring mainly the intestinal environment, or hydrogen, methane, and carbon monoxide. The majority of the Japanese exhale hydrogen only, while it is said that other Asians and Europeans exhale hydrogen and methane.

In digestion and metabolism, acetone and isoprene are gaining attention as metabolic gases. In the past, it was believed that breath acetone reflected the real time glucose level in blood, and much R&D was conducted, but it was found that there was no correlation between acetone and the glucose level. Currently, acetone has been applied to diet control. For example, people worried about body shape have the desire to burn the fat off efficiently but wish to eat as much cake as the fat that was burnt off, and the "harapeko (hungry)" sensor has been developed to measure breath or acetone of the skin responding to this desire.[5] Acetone is an index for burning fat and isoprene is said to be the index for using energy produced by metabolizing the muscles, and the detection of such metabolic gas is in extremely high demand. Research is being conducted actively to clarify the relationships between various diseases and breath components, such as monitoring the liver function using ammonia, evaluation of cholesterol metabolism by isoprene, and monitoring the blood caroboxy hemoglobin by carbon monoxide.

The periodontal disease detector using the composite analysis of breath odor components is already on the market. The measurement of halitosis (bad breath) and the measurement device are used by dentists. The main cause of halitosis is methyl mercaptan that is produced by periodontitis.[6] Sulfur biogas such as methyl mercaptan is also reported to be a marker for colon cancer or liver disease, and the demand for the measurement for this purpose can be expected in the future. Since the dentists can directly see inside the patient's

Category	Chemical formula	Name of gas	Relation to physical condition (literature)	Sensing method
Reducing	H_2	Hydrogen	Abnormality of intestinal anaerobes	Semiconductor (ppm)
	CH_4	Methane	Abnormality of intestinal anaerobes	
	CO	Carbon monoxide	Smoking, oxidation stress	EI-Chem(ppm)
	C_2H_5OH	Ethanol	Drinking	Semiconductor (ppm)
	CH_3COCH_3	Acetone	Diabetes, obesity, dieting	Semiconductor (ppm)
	H_2O_2	Hydrogen peroxide	Smoking	
	C_5H_8	Isoprene	Cholesterol synthesis intermediates	
Sulfur	H_2S	Hydrogen sulfide	Periodontitis	GC/MS(ppb)
	CH_3SH	Methyl mercaptan	Periodontitis, liver disease, colon cancer	GC/MS(ppb)
Amine	NH_3	Ammonia	Hepatitis, *H. Pylori* test	Semiconductor (ppm)
VOC	$C_9H_{18}O$	Nonanal	Lung cancer	GC/MS(ppb)
		Benzene	Lung cancer	

High precision and high response
Hydrogen detection without effect of humidity
Same response to all gases

Fig. 1 Relationship between biogas types and physical conditions or diseases. The sensing methods are included.

mouth, the presence of periodontitis can be determined immediately. However, since many people are conscious of halitosis, there is a demand for halitosis detectors as patients are more satisfied if they can see the decrease of halitosis in gas concentration, before and after the treatment of periodontitis.

Lung cancer is the primary concern in our society, as it is the top cause of death by disease in Japan. The background of high mortality of lung cancer is because it is often too late when lung cancer is diagnosed at the hospital and the patient is notified. If the sign of lung cancer can be detected early, it can be treated by surgery, but there is no good measurement method for early diagnosis. It is hard to find by chest x-rays, and diagnosis is given after sputum analysis and extensive examination with CT. While the technology of breath analysis is a long way from being realized, whenever we see that lung cancer may be detected by smell in the news, we feel that there is high expectation for such technology. In fact, aldehyde VOC is considered to be a marker for lung cancer and particularly there are many studies on nonanal gas.[7] Nonanal is suspected to be related not only to lung cancer but also to other types of cancer, and measurements are made using high-precision analyzers. Nonanal is also said to be the source of body odor of the elderly, but its detection is quite difficult with an ordinary semiconductor sensor due to its high molecular mass.

3 Synthesiological elements in the gas sensor development

3.1 Elemental technologies necessary for breath analysis

At present, it is rare that a patient's biogas is collected, analyzed in the pathology lab, and used for diagnosis in the clinics. However, the number of cases where breath and other gases are analyzed for research are increasing. This new trend is due to the fact that the performance of gas analyzers, as exemplified by the gas chromatography mass spectrometer (GC-MS) that is used most frequently in medical institutions, has greatly improved in terms of speed and accuracy.

As shown in Fig. 2, the general flow of breath analysis is to separate the gas species with GC and to measure the gas concentration with the MS. In some cases, flame ionization detector (FID) that is an easier device to handle may be used. However, for these devices, helium must be used as flow gas, facility may be large, or operators may be necessary and a physician cannot conduct the analysis by him/herself, and we still do not have an environment where the measurement can be done easily. As the demands for the analyses of biogas and environmental gas increase, many gas detector products are being developed using semiconductor sensors and GC to meet these demands. We have developed a similar semiconductor sensor for lung cancer analysis, and the differences and characteristics of ours will be discussed later.

For the wide use of gas analysis, it is necessary that such an analyzer be used easily and readily. In this research, a goal was to realize a breath gas detection system with high performance and accuracy at a level where medical diagnosis is possible, by providing sufficient sensitivity and gas selectivity to a gas sensor element, without the complex pretreatment system. In the simplest structure, as shown in the red dashed line in Fig. 2, the measurement is made directly from the breath without passing through the gas separation mechanism, and a sensor technology that allows both gas selection and highly sensitive detection is necessary

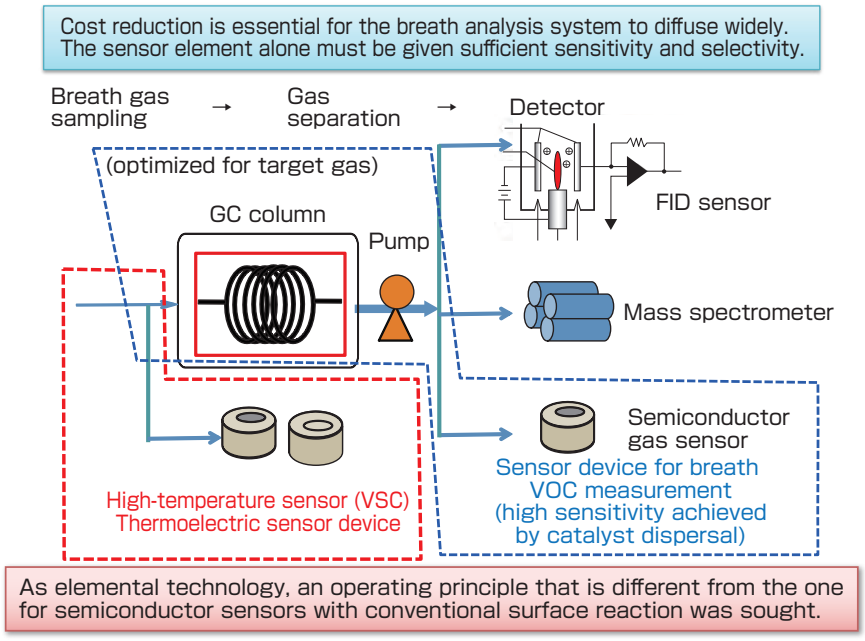

Fig. 2 General flow of the measurement method for breath analysis

for its realization. It was a challenge to create new technology that did not exist before, and it was an opportunity for us to review the weakness of the conventional technology.

3.2 Two main elemental technologies

Two simple breath measurement systems have been developed with the integration of high-temperature ceria sensor and the thermoelectric hydrogen sensor. These two sensor devices can measure the breath gas directly and the systems immediately display the amount of ppb or ppm in about a few tens of seconds.

Methyl mercaptan of sulfur biogas is the main component of halitosis caused by periodontitis. Recently, it has been reported that it may be also related to colon cancer or liver diseases. We succeeded in developing a new sensor that quickly measures volatile sulfur compound (VSC) gas in a highly humid environment. Figure 3 shows the comparison between the mechanism of the high-temperature ceria sensors to detect selectively methyl mercaptan and the principle of conventional surface reaction sensors. The semiconductor sensor that uses the conventional surface reaction is highly sensitive, but is easily affected by humidity and is slow in response. We sought a bulk response type device with different operating principles, and realized a method using the concentration of conductive carrier of the entire material as the gas detection function.

The high-temperature operating ceria sensor that we developed is a bulk response type,[8] and it detects gas by the variation in carrier electron concentration generated from the oxygen vacancy of the electron-conductive oxide. Since the diffusion rate of the oxygen vacancy produced by surface reaction $2Ce_{Ce}^{x} + O_o^{x} \rightarrow 2Ce_{Ce}' + V_O^{\cdot\cdot} + 1/2O_2$ is extremely quick, the change in carrier electron concentration involves the entire bulk, not just the surface. Therefore, it is necessary to raise the sensor temperature, and it is operated at a temperature of 500 ºC or higher. This operating temperature is an important point in the application as a VSC sensor. Since gas species other than VSC undergo oxidation near ceria of the gas sensor thick film due to high temperature, the contribution of ceria to carrier density variation is reduced. Therefore, it was possible for the high-temperature operating ceria sensor to achieve both the selective detection and high sensitivity at ppb level against the VSC gas. The response is fast due to high temperature operation, and this was employed instead of the old sensor. The “II” was added to the product name for differentiation.[9]

For the breath hydrogen which is the index of the intestinal environment, the catalyst of the thermoelectric is maintained at as low as 100 ºC. In the thermoelectric hydrogen sensor, the temperature of the catalyst is kept low so that the combustion catalyst burns hydrogen only. For this hydrogen sensor, its operating principle of converting combustion heat into electric signals was new, and we could conduct the development of new applications such as breath hydrogen measurement with hydrogen concentration of 0~200 ppm at high humidity. This technology does not operate on the principle of resistive semiconductor sensors, but is an efficient method using the small heat of hydrogen combustion and the thermoelectric conversion principle, which can eliminate the effect of humidity or other flammable gases, and therefore, highly sensitive gas detection became possible.[10] The measurement is quick, in one minute per sample since retention time for the separation of gases is unnecessary. We developed a prototype detector that allows simple and easy operation by medical personnel on site by adding functions such as automatic calibration, automatic aspiration, and measurement.

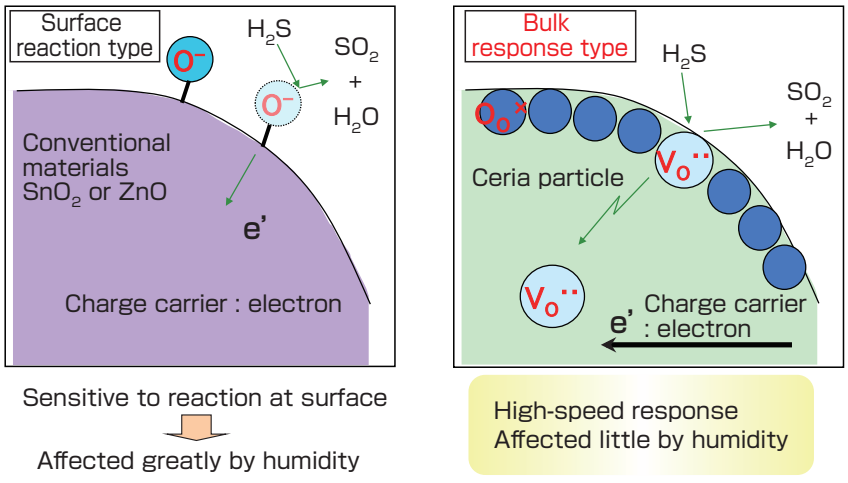

Fig. 3 Even for the same resistive type sensors using oxide semiconductors, the surface reaction and bulk response types have fundamentally different mechanisms, and their reliabilities differ in practice. (O_o^{x} and $V_o^{\cdot\cdot}$ indicate the oxygen in crystals and the oxygen vacancies from which oxygen have escaped.)

3.3 The third technology was system integration of elements

The third gas sensor technology was the sensor for odor (VOC) related to diseases. Commonly, a very expensive and difficult-to-operate analyzer was used to investigate the relationships between the aldehyde VOC and lung cancer or other cancers. However, if the focus is on some specific VOCs, it is not necessary to measure all gas species, and only a few VOCs should be measured. In this case, the issue is which performance should the sensor have. First, the concentration of the odor substance VOC is at the ppb level, and it is necessary for the sensor to have high sensitivity that can measure very low gas concentration. The breath contains a high concentration level of hydrogen, and the sensor must have selectivity against humidity and other various gases. It is extremely difficult to achieve both high sensitivity and high selectivity simultaneously.

Therefore, a system that combines a sensor with GC to separate the gases, as shown in a blue dashed line in Fig. 2, was considered. In the case of a system consisting of simple GC and a sensor, the gas separation mechanism is added by combining with GC, so the sensor must be able to measure all gas species evenly, rather than measure any specific gas selectively. To increase the breath VOC sensor sensitivity, we tried to demonstrate an extremely sensitive gas detector using thick film coating that uses dispersion technology of nanoparticles carrying much catalysts.

To enhance response, we attempted to achieve high sensor sensitivity by developing a dispersion paste of the sensor material and by investigating the relationship between film thickness and sensor response. To fabricate a homogenous paste of sensor material of SnO_2 fine particles carrying Pt, Pd and Au, a dispersion process using a vehicle 'organic dispersion agent' containing ethyl cellulose was developed. By applying and sintering this paste on a substrate, we formed a film with homogenous thickness and pores that allowed diffusion of gas. By optimizing the film thickness for sensor response, the detection of 55 ppb nonanal, a candidate for a lung cancer marker, was achieved.[11] Utilizing this semiconductor sensor and GC separation technology, the ppb level VOC detection in breath was attained.

4 Research result

4.1 Element and boundary condition

Using the halitosis sensor described above, a new detector has been released on the market by greatly improving the response performance of the old model. The hydrogen sensor allows simple breath hydrogen measurement, and has been used in the breath measurements for mass examinations at medical institutions. The breath VOC sensor is being developed as a prototype system for early detection of lung cancer. However, the main point of detector development in this paper is a strategy how to achieve a practical level rather than technical details of each sensor. Figure 4 shows the value or benefit of the product for the user, which are the boundary conditions of the technological component, and the integration of components that were developed for these conditions is organized.

For the halitosis sensor, the old detector using the sensor element with slow response speed requires 45 seconds for sampling and measuring the target gas. It feel like a long time if one actually experiences it. For gas sampling, one

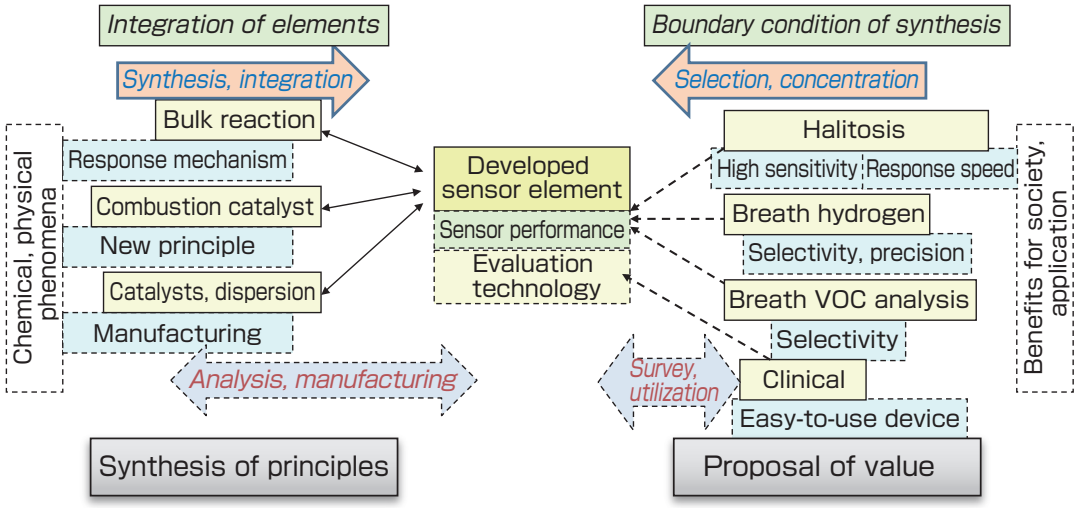

Fig. 4 Correlation diagram of the synthesiological elements in sensor development and the boundary conditions for synthesizing the elements

must hold the tube (mouthpiece) in the mouth and shut the mouth tightly and breathe through the nose, but it is hard to maintain this position for 45 seconds. Without concentration, the mouth eases open or one may start breathing through the mouth. People who experience this test strongly feel the need to shorten the sensor response time. It must be noted that the response speed of the detector and of the sensor are not the same. It is necessary to shorten the response time of the sensor element by enough margin compared to the product on the market.

For the hydrogen sensor, breath hydrogen detectors with the hydrogen sensors were used for quick, easy, and highly precise hydrogen gas concentration measurements at the Aichi Kenko Plaza. Detailed interviews were done of the subjects to clarify the relationship between hydrogen gas concentration and lifestyle and diet, or disease.[12] Measurements of hydrogen concentration was taken from breath gas of about 200 subjects, and the following results were obtained: 1) correlations were found between breath hydrogen gas concentration and age, exercise, intake of milk, hyperlipidemia, and anemia; and 2) hydrogen gas concentration in the breath was apparently high in the group positive for exercise, milk intake, hyperlipidemia, and anemia. Since sufficient number of breath hydrogen concentration measurements has not been collected, further measurements are ongoing. If measurements of over 600 subjects could be obtained, it will be considered as the average value of breath hydrogen concentration for the Japanese (the average breath hydrogen value has not been reported anywhere in the world).

For the lung cancer sensor or the breath VOC detector, the breath before and after surgery of about 200 lung cancer patients at Aichi Cancer Center was analyzed by GC-MS. The breath gas markers were investigated and applied as patents with the system for detecting them. The invention involved the combination of several gas components rather than using just one gas species such as nonanal.

The system combined the GC and the sensor. The sensor should respond equally to various gas species separated by GC and it must be easy to calibrate. The developed system should be user-, physician-friendly, that is, simple enough to be used in the clinical field, and thus contribute to our society.

4.2 Scenario for product realization

In order to release something new through R&D, the synthesiological technological element and the social demand that serves as the boundary condition for their synthesis must be matched. Up to now, the flow was explained in the following four steps.[13] However, for the health care and medical devices, further considerations are necessary.

(1) Idea: discover through new ideation or uncover demands
(2) Fusion of knowledge: specify ideas and demands by quantative experiments
(3) Synthesis: set necessary properties for application goals and run the development
(4) Finalization: summarize the research results and prepare for next research

Substantial resources and large effort are necessary for the R&D of medical devices, and more time and money are needed to confirm the clinical efficacy. However, the actual market is not big enough to pay back such investment. There is extremely high risk for R&D and product realization, and sharp business decisions are necessary. To reduce these risks from the engineer's perspective, it is necessary to develop platform technology.

Taking a bakery as an example, the same technology and machine are used to bake various types of bread. For medical devices of gas sensors, it is necessary to have a solid foundation of micro-heaters, integration technology to add catalysts, ceramic technology that allows catalysts to be applied firmly and well dispersed on the devices, and a gas measurement and evaluation system where the gas can be passed through to evaluate the response. The number of university labs working on the R&D of gas sensor has reduced, and we are the only lab in AIST engaging in this research. It is important for us to keep a strong foothold of this technology.

The final issue in the realization of the device is the collaboration of medicine and engineering. Figure 5 shows the three sensors in three detectors discussed in this paper. The halitosis sensor on the left has been already commercialized. The hydrogen sensor in the middle is scheduled for commercialization in 2016. With the VOC detector on the right, steps toward practical use are on-going, such as verification tests in clinical settings. For the breath hydrogen sensor, the breath of about 600 volunteers have been measured at the Aichi Kenko Plaza since 2014, and the clinical research to study the correlation between the hydrogen concentration and health conditions was conducted. Without this study, the planning of commercialization would have been difficult. Without validation of use in medical institutions, the measurement device will be nothing more than a research facility or a toy.

As a future issue, a method for sampling breath gas must be considered. As shown in Fig. 5, gas sampling methods differ according to the biogas species or disease. In the case of hydrogen, the subjects are instructed to inhale, hold the breath for 2 or 3 seconds, exhale for 1 or 2 seconds, and then the end-tidal breath is collected. For halitosis, on the contrary, the subject is asked to breathe through the nose and the gas in the mouth is suctioned with a pump. In the case of nitric oxide measurement used by asthma patients, the subject blows on the

device at a certain flow rate. Gas samplings from the skin or fecal matter are complicated and there is no clearly established method. From the viewpoint of the research group trying to develop the sensors, it seems that there is no specific research issue, but we believe we will gain new ideas and discoveries in the process of completing this project.

5 Discussion: What follows the scenario

5.1 We must actively propose how the device should be used

As it was mentioned in the scenario for practical application, even if a simple detector is made, the quantification of the relationship between gas concentration and health index or disease is essential for the device to be used in medical practice. Although collaboration with health care and medical fields is important for device application, there is a barrier between the different fields. When excellent technology is obtained through R&D at AIST, we wish to reach the final goal of commercialization. However, in the case of health care and medical businesses, it is difficult to move on to practical use regardless of whether the company which does the development is a small, medium, or major enterprise. Particularly in Japan, we have very little experience in commercialization and do not understand the value chain.

To change this situation, we have founded an AIST consortium of the sensing technology for health care application. It is composed of industrial members of various manufacturing companies such as of materials, measuring device development, medical standard gas, gas sensor, and medical devices, as well as academic members from universities, public institutions, and medical institutes. Along with the industrial members, we are seeking the direction of how we should associate with the medical people in clinics, what kind of demands there are, and what will be necessary in the future. A series of seminars held by the consortium are used as a place to seek the possibilities of collaboration with life science researchers of AIST as well as medicine-engineering collaboration between the different organizations. I hope interesting projects will be born from this.

In the development of new technology, a challenging proposal is important. I have asked the physicians in medical schools, who are members of the joint research, about the case studies of hydrogen gas measurement and the relationship with disease. However, after several disussions, I ended up researching medical papers and creating a summary. It is a very different culture compared to the USA. The medical partners are waiting for the proposal from engineers.

It is expected that a smart phone equipped with electronic nose that smells the air contamination or odor will be marketed in about two or three years. In the future, the medical device will become available as a general home electric appliance or a mobile device. We must accelerate the development for downsizing and achieving higher precision so such devices can be used for health monitoring to find early signs of disease and health management.

6 Conclusion

In the human gas, breath, monitoring technology from which human bioinformation can be obtained non-invasively, decreasing the cost of the system is essential for the spread of the breath analyzing system. In this research, we developed a sensor device and a detector that aimed at a high-performance breath gas detector at the level medical

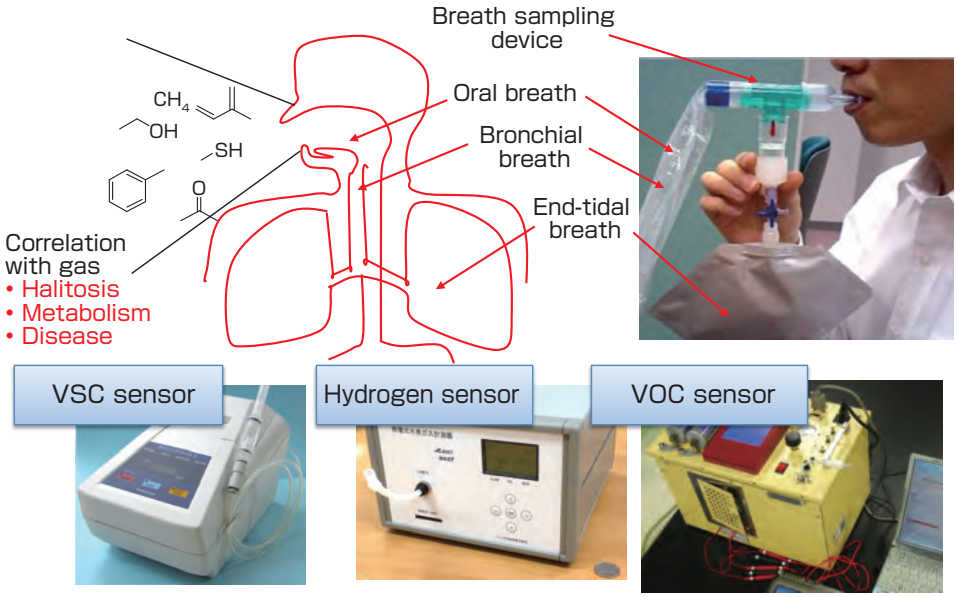

Fig. 5 Summary of the correlation of human biogas and sensor technology used in breath measurement

diagnosis can be made, by providing sufficient sensitivity and gas selectivity to the gas sensor element that does not require a complex pretreatment system. To realize this device, the combination of nanoparticulation technology, paste technology, and small high-temperature heater technology that have been developed in the basic research was important. However, commercialization is difficult with the technologies only, and we must be active in making proposals on what to do to make the device usable for the physicians who will be actually using the detector in clinical application, or what we can know from the breath gas.

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its application technology, and the development of high-temperature operating gas sensor. In this paper, was in charge of the development of halitosis sensor (VSC sensor), and specifically developed the ceria nanoparticle that is the material of the sensor.

Discussions with Reviewers

1 Overall

Comment (Tetsuhiko Kobayashi, AIST)

This paper describes the technology to provide inexpensive health monitoring and medical diagnosis by analyzing the gas components of breath and other gases using the gas sensor. It is not only about the development of the gas analysis system, but it also verifies the usefulness of the developed technology through collaboration with health care and medical institutions. The development is done with strong awareness of social demand, and I believe it is suitable for publication in *Synthesiology*.

Comment (Katsuhiko Kadoguchi , AIST)

Development of medical and measuring devices to determine the health condition and presence of disease easily without affecting the human body is a topic that precisely grasps the demand in the globally-expanding market of the health care and medical treatment. The process of improving the authors' original breath sensor and analytical measures and integrating the elemental techonologies systematically into a product, with the cooperation of medical institutions and volunteers, is worth being depicted synthesiologically.

2 Development method and approach in the medical field (foreign field)

Question (Katsuhiko Kadoguchi)

The present research was carried out with the help of a large number of samples obtained through the cooperation of a medical institution and subjects (patients). On the other hand, in order to ensure the widespread and easily available use of the device developed in this research in the future, it would be most ideal for the researcher to be on site when the physician treats the patients, to observe the work, to understand the instructions and guidance given to the patients, to ethnographically pick up the diagnostic needs that even the physicians themselves may not be aware of, and then to conduct the research with the obtained information taken into consideration. However, this is almost impossible from the viewpoint of the patient's privacy protection, and I assume the difficulty of technological development lies there. I would suppose that understanding the demands through the activity of the consortium tends to be indirect, but how is it actually?

Answer (Woosuck Shin)

In device development, it is important for the researcher to be in company with the physician sampling the patient's breath. In fact, we have communicated to the physicians the proper instructions to give to the patients during breath sampling. The breath sampling of lung cancer patients is done in the examination room, and it is difficult to be there actually. In the case of breath measurement at regular medical checkups for groups, we directly instruct the breath sampling method at the venue. As you indicate, I think it is important to ethnographically collect the needs that the medical practitioners may not be aware of. If we engage in any development by medicine-engineering collaboration in the future, I hope to include this in the research plan. Also, it is impossible for an engineering research group to be in charge. Therefore, collaboration with people within AIST or external organizations is mandatory, and how to create a comprehensive, organic organization will be the point in raising the quality of the R&D.

3 Prospect and future development

Comment (Katsuhiko Kadoguchi)

From the viewpoint of health care, daily self-diagnosis at hospitals or at home can be assumed. In such cases, the device users are not physicians nor nurses but ordinary people. Therefore, I think it is also possible to take an approach of asking cooperative subjects for making a tentative use of the prototype for the development. I think the current technological development targets the use at the site of medical practice. Please give some comments on how the authors intend to correspond to these widespread practical needs in the future, eg. rearrangement of devices for specialist use to those for ordinary use, changing the purpose of the application from treatment of disease to management of pre-disease, etc.

Answer (Woosuck Shin)

I addressed the point that you indicated in the last part of discussion along with the demands accompanying the evolution in recent mobile devices.

4 Detection of abnormality

Question (Katsuhiko Kadoguchi)

How do you treat the individual differences in applying this technology (such as man/woman, adult/child, or height/weight variations, and others)? Also, for an individual, won't the breath component be affected by physical conditions, an empty or full stomach, being fully rested or exhausted, for example?

Answer (Woosuck Shin)

For the medical questions like the ones you asked, pertaining to breath hydrogen gas, an experiment was done with 426 volunteers in FY 2014.

The average value of breath hydrogen was 20.2 ± 21.1 ppm, and there was no sexual difference. The standard range of breath hydrogen (95 % confidence interval of breath hydrogen measurement value) was thought to be 0~79.4 ppm. For breath hydrogen, weak positive correlation was seen in age, and high values were measured in elderly females. For the relationship between breath hydrogen and lifestyle, correlations were seen for items of milk intake, defecation, and exercise. This article has been submitted to *Antei Doitai to Seitai Gas* (Japan Society for Medical Application of Stable Isotope and Biogas) (November 2015).

5 Sensor technology

Comment (Tetsuhiko Kobayashi)

I think the understanding will deepen for the reader who may be a sensor engineer, if you add a little more detailed technological explanation of the sensor. For example, I think you should add the following: 1) the reason why there is selectivity in the VSC sensor, 2) the reason why you can achieve high sensitivity even in the bulk response type VSC sensor, and 3) the merits of using thermoelectric conversion rather than conventional catalyst combustion in the thermoelectric hydrogen sensor.

Answer (Woosuck Shin)

I added the technological explanations of the gas sensors in this paper.

6 Collaboration of different research fields within AIST

Question (Tetsuhiko Kobayashi)

You describe the medicine-engineering collaboration with the external organization, but what do you think about the collaboration between the life science researchers and material/device researchers within AIST?

Answer (Woosuck Shin)

Although we have not reached concrete collaboration, we are seeking collaboration by inviting the life science researchers of AIST to the consortium lectures. I hope some interesting "chemical reaction" will occur soon.

Editorial Policy

Synthesiology Editorial Board

Objective of the journal

The objective of *Synthesiology* is to publish papers that address the integration of scientific knowledge or how to combine individual elemental technologies and scientific findings to enable the utilization in society of research and development efforts. The authors of the papers are researchers and engineers, and the papers are documents that describe, using "scientific words", the process and the product of research which tries to introduce the results of research to society. In conventional academic journals, papers describe scientific findings and technological results as facts (i.e. factual knowledge), but in *Synthesiology*, papers are the description of "the knowledge of what ought to be done" to make use of the findings and results for society. Our aim is to establish methodology for utilizing scientific research result and to seek general principles for this activity by accumulating this knowledge in a journal form. Also, we hope that the readers of *Synthesiology* will obtain ways and directions to transfer their research results to society.

Content of paper

The content of the research paper should be the description of the result and the process of research and development aimed to be delivered to society. The paper should state the goal of research, and what values the goal will create for society (Items 1 and 2, described in the Table). Then, the process (the scenario) of how to select the elemental technologies, necessary to achieve the goal, how to integrate them, should be described. There should also be a description of what new elemental technologies are required to solve a certain social issue, and how these technologies are selected and integrated (Item 3). We expect that the contents will reveal specific knowledge only available to researchers actually involved in the research. That is, rather than describing the combination of elemental technologies as consequences, the description should include the reasons why the elemental technologies are selected, and the reasons why new methods are introduced (Item 4). For example, the reasons may be: because the manufacturing method in the laboratory was insufficient for industrial application; applicability was not broad enough to stimulate sufficient user demand rather than improved accuracy; or because there are limits due to current regulations. The academic details of the individual elemental technology should be provided by citing published papers, and only the important points can be described. There should be description of how these elemental technologies are related to each other, what are the problems that must be resolved in the integration process, and how they are solved (Item 5). Finally, there should be descriptions of how closely the goals are achieved by the products and the results obtained in research and development, and what subjects are left to be accomplished in the future (Item 6).

Subject of research and development

Since the journal aims to seek methodology for utilizing the products of research and development, there are no limitations on the field of research and development. Rather, the aim is to discover general principles regardless of field, by gathering papers on wide-ranging fields of science and technology. Therefore, it is necessary for authors to offer description that can be understood by researchers who are not specialists, but the content should be of sufficient quality that is acceptable to fellow researchers.

Research and development are not limited to those areas for which the products have already been introduced into society, but research and development conducted for the purpose of future delivery to society should also be included.

For innovations that have been introduced to society, commercial success is not a requirement. Notwithstanding there should be descriptions of the process of how the technologies are integrated taking into account the introduction to society, rather than describing merely the practical realization process.

Peer review

There shall be a peer review process for *Synthesiology*, as in other conventional academic journals. However, peer review process of *Synthesiology* is different from other journals. While conventional academic journals emphasize evidential matters such as correctness of proof or the reproducibility of results, this journal emphasizes the rationality of integration of elemental technologies, the clarity of criteria for selecting elemental technologies, and overall efficacy and adequacy (peer review criteria is described in the Table).

In general, the quality of papers published in academic journals is determined by a peer review process. The peer review of this journal evaluates whether the process and rationale necessary for introducing the product of research and development to society are described sufficiently well.

In other words, the role of the peer reviewers is to see whether the facts necessary to be known to understand the process of introducing the research finding to society are written out; peer reviewers will judge the adequacy of the description of what readers want to know as reader representatives.

In ordinary academic journals, peer reviewers are anonymous for reasons of fairness and the process is kept secret. That is because fairness is considered important in maintaining the quality in established academic journals that describe factual knowledge. On the other hand, the format, content, manner of text, and criteria have not been established for papers that describe the knowledge of "what ought to be done." Therefore, the peer review process for this journal will not be kept secret but will be open. Important discussions pertaining to the content of a paper, may arise in the process of exchanges with the peer reviewers and they will also be published. Moreover, the vision or desires of the author that cannot be included in the main text will be presented in the exchanges. The quality of the journal will be guaranteed by making the peer review process transparent and by disclosing the review process that leads to publication.

Disclosure of the peer review process is expected to indicate what points authors should focus upon when they contribute to this journal. The names of peer reviewers will be published since the papers are completed by the joint effort of the authors and reviewers in the establishment of the new paper format for *Synthesiology.*

References

As mentioned before, the description of individual elemental technology should be presented as citation of papers published in other academic journals. Also, for elemental technologies that are comprehensively combined, papers that describe advantages and disadvantages of each elemental technology can be used as references. After many papers are accumulated through this journal, authors are recommended to cite papers published in this journal that present similar procedure about the selection of elemental technologies and the introduction to society. This will contribute in establishing a general principle of methodology.

Types of articles published

Synthesiology should be composed of general overviews such as opening statements, research papers, and editorials. The Editorial Board, in principle, should commission overviews. Research papers are description of content and the process of research and development conducted by the researchers themselves, and will be published after the peer review process is complete. Editorials are expository articles for science and technology that aim to increase utilization by society, and can be any content that will be useful to readers of *Synthesiology.* Overviews and editorials will be examined by the Editorial Board as to whether their content is suitable for the journal. Entries of research papers and editorials are accepted from Japan and overseas. Manuscripts may be written in Japanese or English.

Required items and peer review criteria (January 2008)

	Item	Requirement	Peer Review Criteria
1	Research goal	Describe research goal ("product" or researcher's vision).	Research goal is described clearly.
2	Relationship of research goal and the society	Describe relationship of research goal and the society, or its value for the society.	Relationship of research goal and the society is rationally described.
3	Scenario	Describe the scenario or hypothesis to achieve research goal with "scientific words".	Scenario or hypothesis is rationally described.
4	Selection of elemental technology(ies)	Describe the elemental technology(ies) selected to achieve the research goal. Also describe why the particular elemental technology(ies) was/were selected.	Elemental technology(ies) is/are clearly described. Reason for selecting the elemental technology(ies) is rationally described.
5	Relationship and integration of elemental technologies	Describe how the selected elemental technologies are related to each other, and how the research goal was achieved by composing and integrating the elements, with "scientific words".	Mutual relationship and integration of elemental technologies are rationally described with "scientific words".
6	Evaluation of result and future development	Provide self-evaluation on the degree of achievement of research goal. Indicate future research development based on the presented research.	Degree of achievement of research goal and future research direction are objectively and rationally described.
7	Originality	Do not describe the same content published previously in other research papers.	There is no description of the same content published in other research papers.

Instructions for Authors

"Synthesiology" Editorial Board
Established December 26, 2007
Revised June 18, 2008
Revised October 24, 2008
Revised March 23, 2009
Revised August 5, 2010
Revised February 16, 2012
Revised April 17, 2013
Revised May 9, 2014
Revised April 1, 2015
Revised October 1, 2015

1 Types of articles submitted and their explanations

The articles of *Synthesiology* include the following types:
• Research papers, commentaries, roundtable talks, and readers' forums
Of these, the submitted manuscripts of research papers and commentaries undergo review processes before publication. The roundtable talks are organized, prepared, and published by the Editorial Board. The readers' forums carry writings submitted by the readers, and the articles are published after the Editorial Board reviews and approves. All articles must be written so they can be readily understood by the readers from diverse research fields and technological backgrounds. The explanations of the article types are as follows.

① Research papers
A research paper rationally describes the concept and the design of R&D (this is called the scenario), whose objective is to utilize the research results in society, as well as the processes and the research results, based on the author's experiences and analyses of the R&D that was actually conducted. Although the paper requires the author's originality for its scenario and the selection and integration of elemental technologies, whether the research result has been (or is being) already implemented in society at that time is not a requirement for the submission. The submitted manuscript is reviewed by several reviewers, and the author completes the final draft based on the discussions with the reviewers. Views may be exchanged between the reviewers and authors through direct contact (including telephone conversations, e-mails, and others), if the Editorial Board considers such exchange necessary.

② Commentaries
Commentaries describe the thoughts, statements, or trends and analyses on how to utilize or spread the results of R&D to society. Although the originality of the statements is not required, the commentaries should not be the same or similar to any articles published in the past. The submitted manuscripts will be reviewed by the Editorial Board. The authors will be contacted if corrections or revisions are necessary, and the authors complete the final draft based on the Board members' comments.

③ Roundtable talks
Roundtable talks are articles of the discussions or interviews that are organized by the Editorial Board. The manuscripts are written from the transcripts of statements and discussions of the roundtable participants. Supplementary comments may be added after the roundtable talks, if necessary.

④ Readers' forums
The readers' forums include the readers' comments or thoughts on the articles published in *Synthesiology*, or articles containing information useful to the readers in line with the intent of the journal. The forum articles may be in free format, with 1,200 Japanese characters or less. The Editorial Board will decide whether the articles will be published.

2 Qualification of contributors

There are no limitations regarding author affiliation or discipline as long as the content of the submitted article meets the editorial policy of *Synthesiology*, except authorship should be clearly stated. (It should be clearly stated that all authors have made essential contributions to the paper.)

3 Manuscripts

3.1 General

3.1.1 Articles may be submitted in Japanese or English.
Accepted articles will be published in *Synthesiology* (ISSN 1882-6229) in the language they were submitted. All articles will also be published in *Synthesiology - English edition* (ISSN 1883-0978). The English edition will be distributed throughout the world approximately four months after the original *Synthesiology* issue is published. Articles written in English will be published in English in both the original *Synthesiology* as well as the English edition. Authors who write articles for *Synthesiology* in Japanese will be asked to provide English translations for the English edition of the journal within 2 months after the original edition is published.
3.1.2 Research papers should comply with the structure and format stated below, and editorials should also comply with the same structure and format except subtitles and abstracts are unnecessary.
3.1.3 Research papers should only be original papers (new literary work).
3.1.4 Research papers should comply with various guidelines of

research ethics

3.2 Structure

3.2.1 The manuscript should include a title (including subtitle), abstract, the name(s) of author(s), institution/contact, main text, and keywords (about 5 words).
3.2.2 Title, abstract, name of author(s), keywords, and institution/contact shall be provided in Japanese and English.
3.2.3 The manuscript shall be prepared using word processors or similar devices, and printed on A4-size portrait (vertical) sheets of paper. The length of the manuscript shall be, about 6 printed pages including figures, tables, and photographs.
3.2.4 Research papers and editorials shall have front covers and the category of the articles (research paper or editorial) shall be stated clearly on the cover sheets.
3.2.5 The title should be about 10-20 Japanese characters (5-10 English words), and readily understandable for a diverse readership background. Research papers shall have subtitles of about 15-25 Japanese characters (7-15 English words) to help recognition by specialists.
3.2.6 The abstract should include the thoughts behind the integration of technological elements and the reason for their selection as well as the scenario for utilizing the research results in society.
3.2.7 The abstract should be 300 Japanese characters or less (125 English words). The Japanese abstract may be omitted in the English edition.
3.2.8 The main text should be about 9,000 Japanese characters (3,400 English words).
3.2.9 The article submitted should be accompanied by profiles of all authors, of about 200 Japanese characters (75 English words) for each author. The essential contribution of each author to the paper should also be included. Confirm that all persons who have made essential contributions to the paper are included.
3.2.10 Discussion with reviewers regarding the research paper content shall be done openly with names of reviewers disclosed, and the Editorial Board will edit the highlights of the review process to about 3,000 Japanese characters (1,200 English words) or a maximum of 2 pages. The edited discussion will be attached to the main body of the paper as part of the article.
3.2.11 If there are reprinted figures, graphs or citations from other papers, prior permission for citation must be obtained and should be clearly stated in the paper, and the sources should be listed in the reference list. A copy of the permission should be sent to the Publishing Secretariat. All verbatim quotations should be placed in quotation marks or marked clearly within the paper.

3.3 Format

3.3.1 The headings for chapters should be 1, 2, 3…, for subchapters, 1.1, 1.2, 1.3…, for sections, 1.1.1, 1.1.2, 1.1.3, for subsections, 1.1.1.1, 1.1.1.2, 1.1.1.3.
3.3.2 The chapters, subchapters, and sections should be enumerated. There should be one line space before each paragraph.
3.3.3 Figures, tables, and photographs should be enumerated. They should each have a title and an explanation (about 20-40 Japanese characters or 10-20 English words), and their positions in the text should be clearly indicated.
3.3.4 For figures, image files (resolution 350 dpi or higher) should be submitted. In principle, the final print will be in black and white.
3.3.5 For photographs, image files (resolution 350 dpi or higher) should be submitted. In principle, the final print will be in black and white.
3.3.6 References should be listed in order of citation in the main text.

Journal – [No.] Author(s): Title of article, *Title of journal* (italic), Volume(Issue), Starting page-Ending page (Year of publication).
Book – [No.] Author(s): *Title of book* (italic), Starting page-Ending page, Publisher, Place of Publication (Year of publication).
Website – [No.] Author(s) name (updating year): Title of a web page, Name of a website (The name of a website is possible to be omitted when it is the same as an author name), URL, Access date.

4 Submission

One printed copy or electronic file (Word file) of manuscript with a checklist attached should be submitted to the following address:

Synthesiology Editorial Board
c/o Public Relations Information Office, Planning Headquarters, National Institute of Advanced Industrial Science and Technology(AIST)
Tsukuba Central 1 , 1-1-1 Umezono, Tsukuba 305-8560
E-mail: synthesiology-ml@aist.go.jp
The submitted article will not be returned.

5 Proofreading

Proofreading by author(s) of articles after typesetting is complete will be done once. In principle, only correction of printing errors are allowed in the proofreading stage.

6 Responsibility

The author(s) will be solely responsible for the content of the contributed article.

7 Copyright

The copyright of the articles published in "*Synthesiology*" and "*Synthesiology English edition*" shall belong to the National Institute of Advanced Industrial Science and Technology(AIST).

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Research papers

Letter from the editor

We bring you the final issue of Volume 8 that is also the last issue of this fiscal year.

The objective of *Synthesiology* is to describe the course by which the result of basic research can be utilized in society by presenting the actual case studies, and to accumulate such case studies as knowledge. In general, to realize a technology, in addition to the integration of multiple elemental technologies from different fields toward a certain goal, it is necessary to set the conditions where the technology may be accepted in society, including the value chain and social acceptance. It is difficult for a single research group to accomplish all these activities, and the cooperation of multiple organizations with different specialties is essential. How such collaboration can be built is the key to wide diffusion of technology in society.

The five papers published in this issue address diverse subjects, from atmospheric scanning electron microscopy, pressworking, internal combustion engine, health care sensing, to structural analysis of biomolecules. However, all of these developments were not the results achieved solely by AIST and the cooperation of other organizations was importan. On the other hand, there are many modes of collaborative relationship. In this issue, good examples of collaboration are presented in the paper that describes the success in advanced pressworking through the cooperation of a medium-sized company that has close contact with the demands on site and AIST that possess basic technology, and the paper about the collaboration of three parties to commercialize a system to estimate the biomolecular structure from mass spectrometry data.

Effective integration of multiple technologies will become necessary to quickly respond to diverse issues. As the need for open innovation increases in reality, where the capacities of external organizations are employed as part of the corporate development strategy, the research institution such as AIST must also strengthen its function as a bridge. I hope the readers see hints for innovative collaboration from the papers in this issue.

(Toshihiko KANAYAMA, Editor in Chief)

Aim of *Synthesiology*
— Utilizing the fruits of research for social prosperity —

There is a wide gap between scientific achievement and its utilization by society. The history of modern science is replete with results that have taken life-times to reach fruition. This disparity has been called the *valley of death*, or the *nightmare stage*. Bridging this difference requires scientists and engineers who understand the potential value to society of their achievements. Despite many previous attempts, a systematic dissemination of the links between scientific achievement and social wealth has not yet been realized.

The unique aim of the journal *Synthesiology* is its focus on the utilization of knowledge for the creation of social wealth, as distinct from the accumulated facts on which that wealth is engendered. Each published paper identifies and integrates component technologies that create value to society. The methods employed and the steps taken toward implementation are also presented.

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Highlights of the Papers in *Synthesiology*

Research papers

"Synthesiology-English edition" is a translated version of "Synthesiology," which is published quarterly, ISSN 1882-6229, by AIST. Papers or articles published in "Synthesiology-English edition" appear approximately four months after the publication of the original "Synthesiology."

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