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The Marriage of Electron Microscopy and Modern Data Science: Entering a New Era in the Study of Materials

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1. Electron Microscopy and the Study of Materials

There are innumerable examples in which the macroscopic properties and performance of a material are governed by its microscopic structure; for this reason, electron microscopes, which offer various kinds of characterization techniques with high spatial resolution[1], have made crucial contributions to the study of materials. To list just a few examples: the mechanical properties of structural materials have been elucidated by in-situ observations of crystal defects via ultra-high-voltage electron microscopy and similar techniques; advanced semiconductor devices have been characterized by localized cross-sectional observations using focused ion beam (FIB) and microsampling techniques; scanning electron microscopy (SEM) has become a critical tool for manufacturing semiconductor devices; electron holography has been used to visualize quantum-mechanical phenomena in superconductors and other exotic materials; cryo-electron microscopy (cryo-EM, for which the 2017 Nobel Prize in Chemistry was awarded) has been used to reveal the structure and function of proteins and biomolecules; and environmentally-controlled transport systems for anaerobic samples have become common tools for characterizing lithium secondary battery materials. A glance at the historical evolution of electron microscopy shows that the needs of advanced material developers have consistently driven advances in characterization techniques—and that cutting-edge microscopic techniques, just as consistently, have spurred progress in materials research and development. The history also clearly shows that Hitachi and Hitachi High-Tech have made seminal contributions to many of the most important technological breakthroughs, including those mentioned above: ultra-high-voltage electron microscopy, FIB techniques, microsampling, critical dimension (CD) SEM, field-emission electron guns, electron holography, and more.

Electron microscopes may be instruments for observing the *physically* small, but the results of these observations often reveal new insights that are *conceptually* enormous; the opportunity to see unexpected new microscopic structures or atomic arrangements *with one's own eyes* often provides scientists researching novel materials and engineers designing advanced devices with clues for a fundamental understanding of the materials. Discoveries of unexpected new materials, structures, or phenomena are often announced in journal publications trumpeting *a successful R&D program targeting just this finding* or *an innovative new conceptual proposal*; this phrasing may seem little more than a self-serving interpretation, but in fact such unexpected results are a typical form of serendipity, and advanced characterization techniques accelerate materials innovation.

2. The Explosion of Electron-microscopy Data—and the Need for Data Science

Up until the early 1990s, electron-microscopy images were exposed on photographic film; researchers would select 20 or so images at a time to develop in a darkroom. In the years that followed, improvements in the performance of system components (detectors, electron guns, spherical aberration correctors, electron spectrometers, monochromators, X-ray detectors, in-situ observation holders, and more), combined to ensure that the data acquired by electron microscopes became multi-modal—and vastly more voluminous. There were even attempts (such as RDE[2]) to convert measurement

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data into structured formats and save it for later use. Recent researchers need to control complicated scientific instruments, then sift through the resulting big data to extract meaningful information; as a result, basic skills in software engineering and data science have become essential prerequisites for *every* stage of materials characterization, from experimental planning to decision-making. Those of us who were educated in earlier eras will protest that we were taught to respect the hardware and revere the raw data above all. Accurate though this may be—and this is my personal opinion with a sense of self-admonition—to my mind it is still no excuse for lacking, in today's world, a solid grounding in data science and numerical analysis.

Indeed, to the modern researcher, electron microscopes and other advanced measurement instruments are more than just metrology systems—they are *platforms for research and development*. As long as instrument vendors continue to provide users with open software development environments accompanying their instruments, we can be sure that forward-thinking researchers and ambitious startup companies around the world will continue to develop new tools to enhance materials characterization.

3. Data Science as a Mandatory Component of Basic Literacy for Modern Researchers

In my student days, I joined a research group in which high-level technical staff was developing laboratory instruments under professors' supervision. That department of university had access to a workshop, which—to my amazement—successfully fabricated an ultra-high-vacuum component from a rather incompetent sketch I had made. By fusing *science* with *engineering*, we were able to do cutting-edge research; I learned that the pace of R&D can be accelerated by a commitment to testing each and every idea—no matter how big or how small—as soon as possible. To my mind, the research organizations most likely to survive and thrive going forward are the ones that implement and test new ideas at early stages—and here I have in mind not just ideas for fabricating and observing samples, but also ideas for improving laboratory instruments, for control systems, for analysis software, and so forth.

I would like to share a topic from my research that illustrates the marriage of electron microscopy and data science. Recent years have seen growing interest in 4D-STEM, a form of scanning transmission electron microscopy (STEM) that captures many diffraction patterns while scanning the incident probe[3)]. Our research group had applied nonnegative matrix factorizations (a dimensionality-reduction technique used in machine learning) to analyze large numbers of diffraction patterns[4)]; this yields a small collection of diffraction patterns from which the full set of experimentally-measured diffraction patterns can be approximately reconstructed, and we used this approach successfully to detect nanocrystalline precipitates in a metallic glass, identifying the crystal structure and the mean grain size[5)]. When we began this research project, we asked a colleague in another research group to develop the computational code; this seemed faster and less expensive than outsourcing the coding to an external contractor. However, we quickly found ourselves inundated with new ideas we wanted to try, and would have felt entirely too *guilty* asking him to develop the code because most of our ideas wound up not working well. Thus, before long I bowed to the inevitable and began developing the code myself. This significantly accelerated the "Plan-Do-Check" loop of our research, in which we would implement a new idea, test its effectiveness, then connect it to a follow-up idea; developing the code myself also enhanced my understanding of existing machine-learning libraries (such as Scikit-learn), and at present we are studying the possibility of adding constraints about electron microscopy. We plan to distribute our code freely via GitHub and other open-source repositories to allow other researchers to benefit from our work. Within the machine-learning community, specialized knowledge possessed by members of outside research disciplines—like us—is called *domain knowledge*; going forward, it seems certain that R&D initiatives to embed domain knowledge within existing machine learning will proceed apace throughout a broad range of research fields.

Incidentally, it is amusing to note that research on non-negative matrix factorizations seems to have begun in earnest after the publication of a Nature article in 1999[6)]—a date that I, now aged 60, cannot help but think of as rather recent. Of course, the technique is ultimately based on the linear algebra that I supposedly learned as an undergraduate some 40 years ago—and which I am currently in the process of reviewing. Around 10 years ago, I was complaining that, for any given topic or paper of interest, "there are hopelessly too many related papers—it's *impossible* to keep up." In response,

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a professor I admired told me: "Studying and learning are lifelong pursuits" which made me reflect on my attitude: if even this *genius* would need to keep studying throughout his life, then *my* brain would require studies lasting well beyond death. Meanwhile, another colleague I respect insists that "researchers these days don't study *nearly* enough." If we do not update our knowledge continuously, our research activities are not sustainable. I am currently affiliated with a research center populated both by researchers studying advanced materials characterization and researchers studying materials informatics[7,8], and the need for characterization researchers to acquire interdisciplinary data-science skills—as a component of basic literacy—has never seemed to me more urgent than it is today.

4. Conclusions

The 2024 Nobel prizes in both physics and chemistry recognized work related to artificial intelligence (AI). The significance of this historical confluence seems clear: In research fields—very much including the study of materials and the study of characterization techniques—the question of how to incorporate AI is *the* central challenge of our time. In the years to come, advanced measurement systems will continue to produce multi-dimensional datasets of ever greater enormousness, thus requiring a continuing infusion of machine-learning techniques to handle the data explosion; consequently, this infusion will not be a one-time transient event, but will instead evolve into a long-term process of entrenchment. Electron microscopes play a key role as advanced scientific measurement instruments, but require precise alignments to ensure correct results; this is one area in which the infusion of AI techniques promises significant advances in coming years. Ever since the invention of the electron microscope by Ernst Ruska (recipient of the 1986 Nobel Prize in Physics) in 1931—and continuing right up to the present—the field has witnessed continual technological progress on many simultaneous fronts. I am confident that the fusion of advanced electron microscopy techniques with modern methods of data science, and the further incorporation of other disciplines through open R&D environments, promises a wealth of unexpected discoveries driving material innovation.

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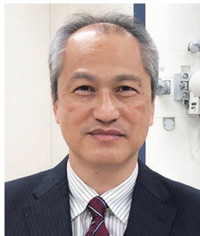
Uranium-free Metal Staining Allows Application of Compact Low-vacuum SEM to Medical Research and Clinical Diagnosis —Developing an Electronic Stain that Converts Paraffin Sections for Light Microscopy to Electron Microscopy Specimens—



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1. Introduction

The heart, kidneys, liver, and other organs that keep us alive have an intricate three-dimensional structure made of cells and connective tissues that confer their physiological function. Breakdown of these cells and tissues leads to a loss of function and normal condition, finally turning into abnormal pathology and resulting in the development of disease. Light microscopes are the main tools used for histopathological diagnosis, which seeks to unlock the etiology of such diseases in the form of morphological changes, but electron microscopy is needed to investigate morphological changes in fine detail. Nevertheless, the latter technique is complicated by the difficult and time-consuming procedures needed for sample preparation and the need for electronic staining, which requires strictly-regulated uranium compounds. A pressing goal for R&D efforts into regenerated organs is the recreation of their three-dimensional structure. Achieving this goal will require convenient and rapid sample preparation techniques for capturing the fine-grained structure of cultured cells and the three-dimensional structure of the insides of organs in intricate detail, and also microscopic techniques useful for screening the quality of regenerated organs.

To solve these problems, we have been working on a new electronic staining procedure that does not use uranium compounds, which has been a long-standing challenge, and successfully established a protocol for conveniently and rapidly imaging the fine-grained morphology of the cells and tissues that make up organs in intricate detail at electron-microscopy resolution. This article discusses the new direction in which tabletop low-vacuum scanning electron microscopes are headed, allowing wide-ranging biomedical and clinical applications such as the histopathological diagnosis of tissue and evaluation of the morphological quality of regenerated organs.

2. Convenient Microscope Slide Holder and Charge-reducing Low-vacuum Mode

The TM4000PlusII tabletop scanning electron microscope is, as its name suggests, a compact instrument that fits on a standard desk. It features a holder for light microscope slides with paraffin sections (Figure 1). Slides can be easily placed on and removed from the holder and, provided paraffin sections 5 to 30 µm in thickness are used, no troublesome height adjustment is required. By saving image files with information about the position where the images were obtained, the holder can return to the same position each time a slide is reinserted.

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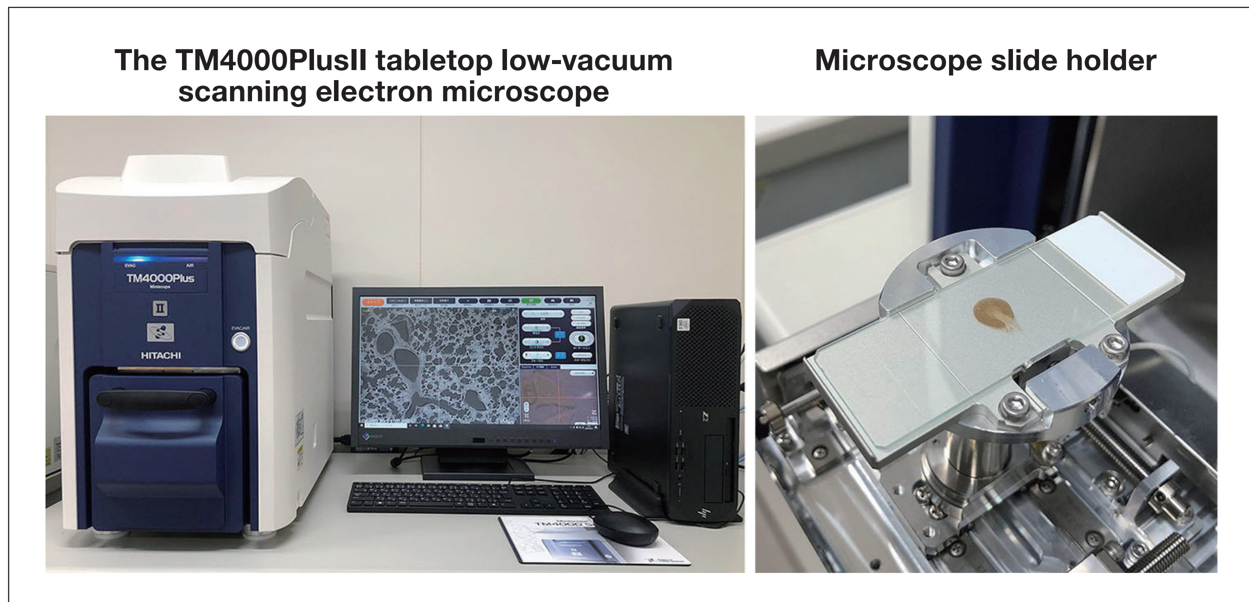

Fig. 1 TM4000PlusII tabletop scanning electron microscope (left). Slide holder (right). Slides can be easily placed and removed, and no height adjustment is required.

The TM4000PlusII operates at a low vacuum of 30 to 50 Pa, so that negative charge accumulated on the nonconductive paraffin section and slide as a result of electron irradiation is neutralized by positively charged ions generated in the remaining gas. This reduces the electrostatic charge that hinders observation (Figure 2).

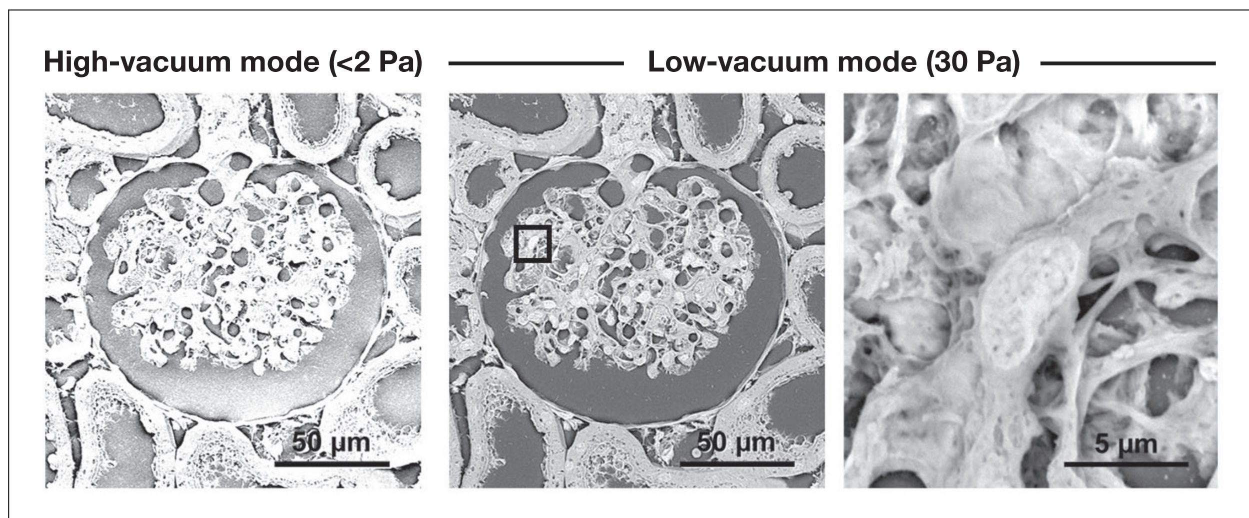

Fig. 2 Micrographs illustrating the difference between high- and low-vacuum modes. Rat renal corpuscle. High-vacuum mode (<2 Pa) (left). High electrostatic charge prevents analysis. Low-vacuum mode (30 Pa) (center and right). With the electrostatic charge greatly reduced, the area within the square in the center micrograph can be analyzed at high magnification (right).

3. Developing an Electronic Stain That Converts Paraffin Sections for Light Microscopy to Electron Microscopy Specimens

The electronic staining technique now widely used was first developed by Watson in 1958, and is based on uranyl acetate and lead[1,2]. However, uranium compounds are strictly regulated, and all steps of the process—from purchase to storage, use, and disposal—are quite complicated. Electron microscopists have long desired a new electronic staining technique that does not require uranium compounds. In the potassium permanganate/lead staining protocol that we developed[3], paraffin sections for light microscopy are treated with an aqueous solution of 0.2% potassium permanganate for 5 minutes, washed with water, treated with Reynolds' lead citrate solution for 3 minutes, and then washed with water and dried. The resulting specimens are ready for observation (Figure 3). Our protocol allows

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convenient and rapid sample preparation, and differs from the conventional technique only in that 0.2% potassium permanganate is used instead of 2% uranyl acetate. The time required for electronic staining remains the same, at about 10 minutes.

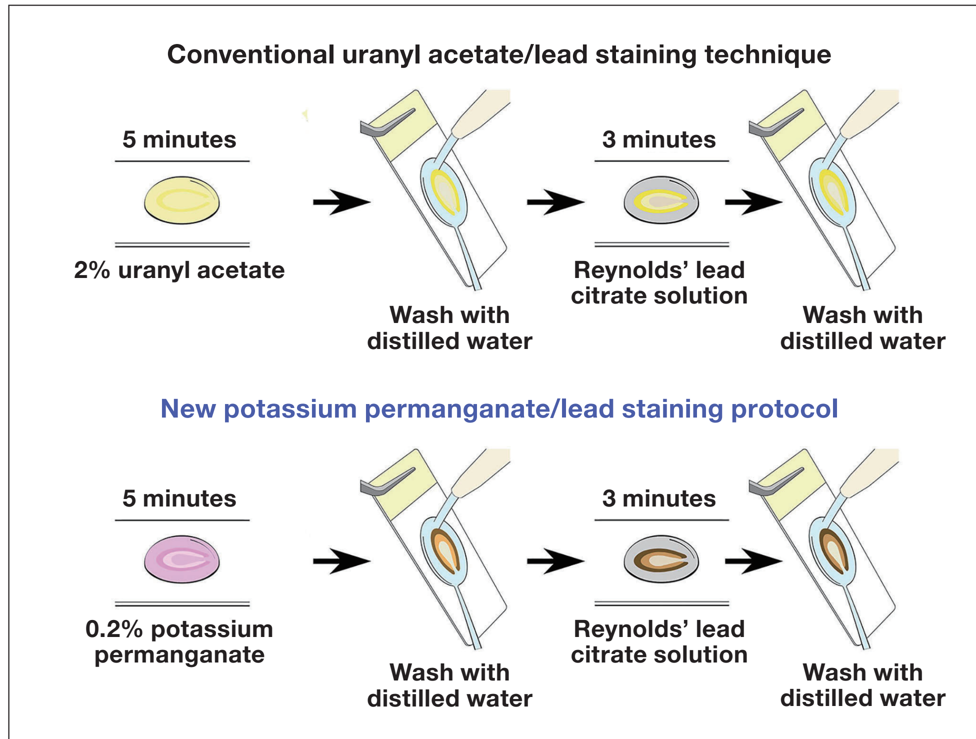

Fig. 3 Conventional uranyl acetate/lead staining technique (top) and new potassium permanganate/lead staining protocol (bottom)

The new potassium permanganate/lead staining method provides high-contrast staining comparable to that for the conventional uranyl acetate/lead staining technique. The attractiveness of the ability to convert samples prepared for light microscopy into samples for electron microscopy, and the power of low-vacuum scanning electron microscopy, are obvious. Elemental analysis showed that lead deposition enhanced by potassium permanganate oxidation resulted in a backscattered electron intensity sufficient to make the fine-grained structures of cells and tissues visible[3].

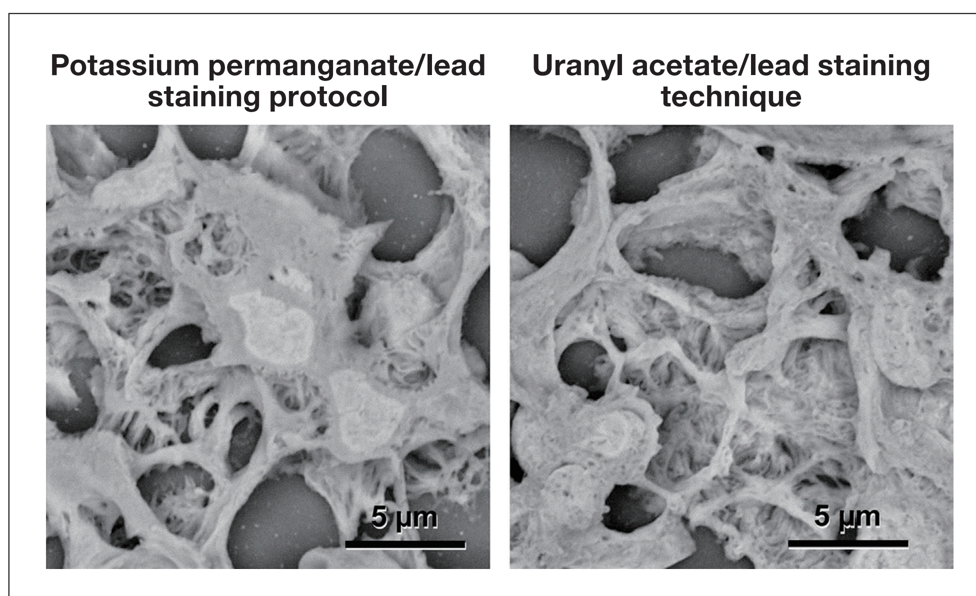

Fig. 4 Rat renal glomerulus. This side-by-side comparison shows tissue stained with the new potassium permanganate/lead staining protocol (left) and conventional uranyl acetate/lead staining technique (right). This comparison highlights the attractiveness of the ability to convert samples prepared for light microscopy into samples for electron microscopy and the power of low-vacuum scanning electron microscopy. Fixing fluid: 2% paraformaldehyde + 2.5% glutaraldehyde. Section thickness: 5 µm.

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Paraffin sections are generally observed in backscattered-electron mode. Since the resulting micrographs differ depending on the accelerating voltage, the voltage must be adjusted to suit the objective of the microscopy observations. Figure 5 shows micrographs obtained at four different accelerating voltages ranging from 5 to 20 kV. Note that low-contrast but clear images of the cell surface can be obtained at low accelerating voltages, whereas high-contrast but unclear images are obtained at high accelerating voltages. Based on our experience, we recommend a voltage of 15 to 20 kV for low-magnification (<500×) microscopy for clearly imaging the overall tissue, and a voltage of 5 to 10 kV for high-magnification (≥500×) microscopy for imaging the intricate architecture of cell surfaces.

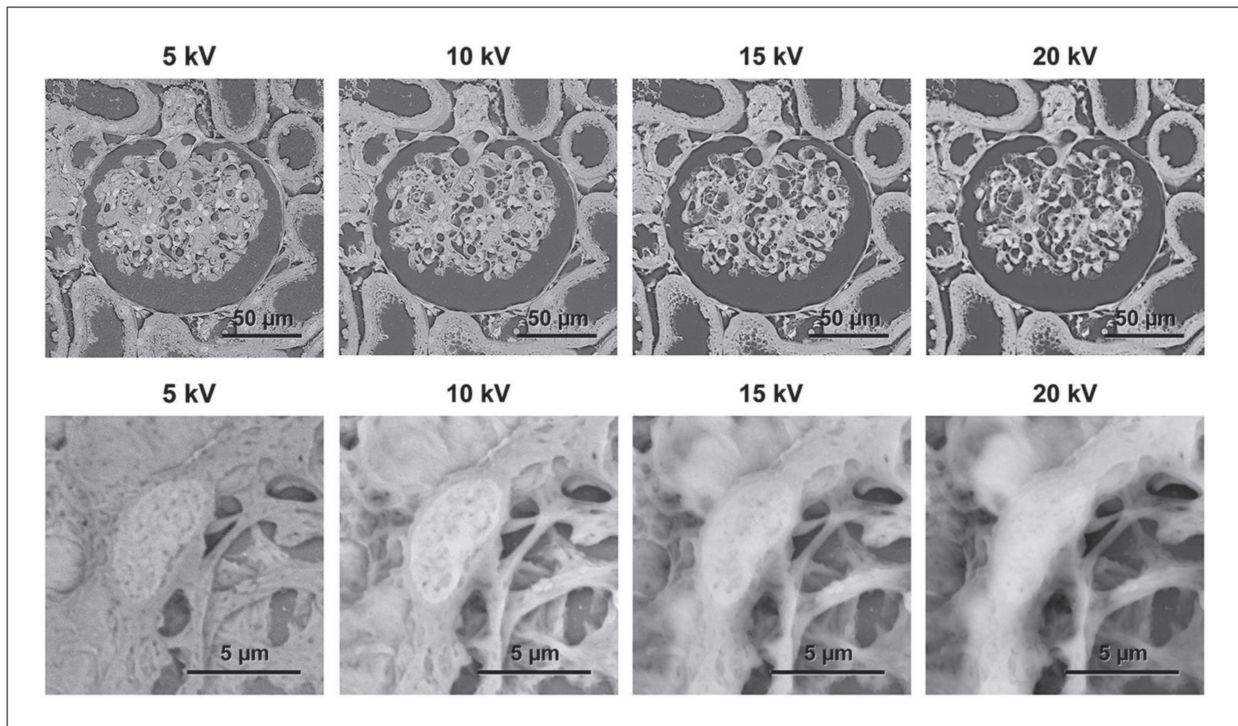

Fig. 5 Micrographs obtained at different accelerating voltages. Note how a low accelerating voltage reduces the contrast but makes the cell surface architecture clear, while a high accelerating voltage increases the contrast but obscures the architecture. We recommend using a voltage of 15 to 20 kV for low-magnification (<500×) microscopy and 5 to 10 kV for high-magnification (≥500×) microscopy.

4. Imaging the Three-dimensional Structure of Cells and Tissues Using Thick Paraffin Section Microscopy

Paraffin sections for light microscopy are typically cut to a thickness of only around 5 µm so as to be permeable to light. Cell nuclei do not overlap at this thickness. However, we previously reported the use of low-vacuum scanning electron microscopy with thick paraffin sections (15 to 30 µm)[2], based on the detection of backscattered electrons. The greater sample thickness allows the three-dimensional structure of tissue cells to be imaged. Figure 6 shows images obtained from thick sections of the rat renal glomerulus, renal tubules, and the ciliated epithelium of the bronchiole stained using the potassium permanganate/lead staining protocol. Although these micrographs were obtained using a tabletop scanning electron microscope, they successfully capture fine cilia and other three-dimensional details not readily imaged with light microscopy.

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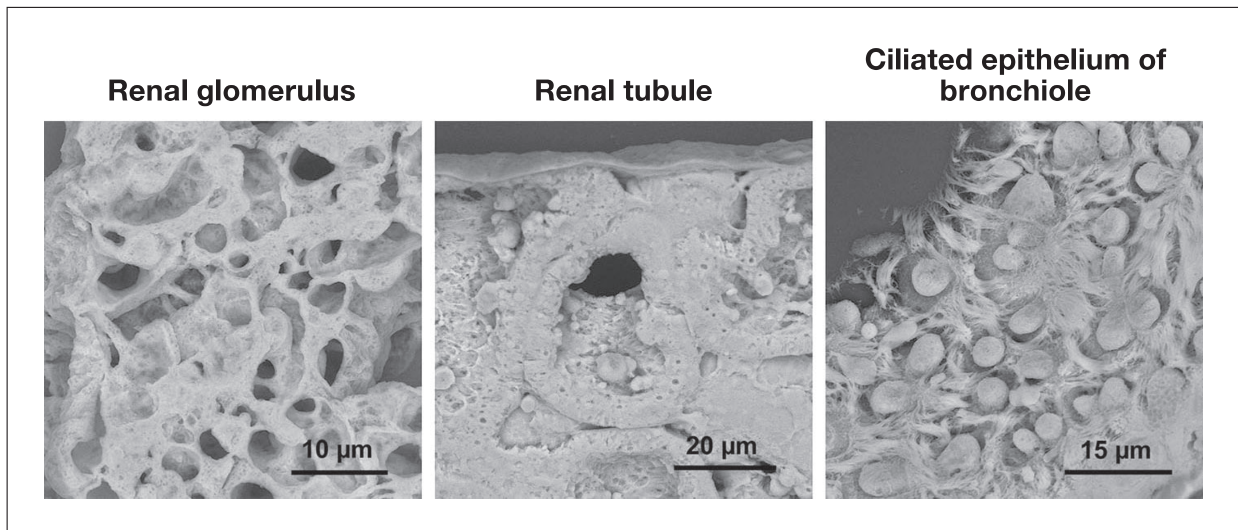

Fig. 6 Thick paraffin section micrographs of rat organs. Cutting sections to 20 µm rather than the standard 5-µm thickness gives the specimens depth, allowing clear imaging of the three-dimensional structure of tissue cells. Fixing fluid: 2% paraformaldehyde + 2.5% glutaraldehyde. Section thickness: 20 µm.

5. Tissue Histopathology Specimens Fixed in Formalin Alone Are Still Imaged in Beautiful, Elaborate Detail

All of the electron microscopy samples described so far were fixed using a standard half-strength Karnovsky's fixing fluid (2% paraformaldehyde + 2.5% glutaraldehyde). Standard histopathology specimens, however, are normally fixed using a 10% formalin solution (4% paraformaldehyde alone). Figure 7 show a scanning electron microscopy image of rat bronchioles fixed using this solution. The image clearly shows the elaborate architecture of the ciliated epithelium and collagen fibers with a crisscrossed, layered structure. This demonstrates that our protocol is suitable for observation of histopathology specimens.

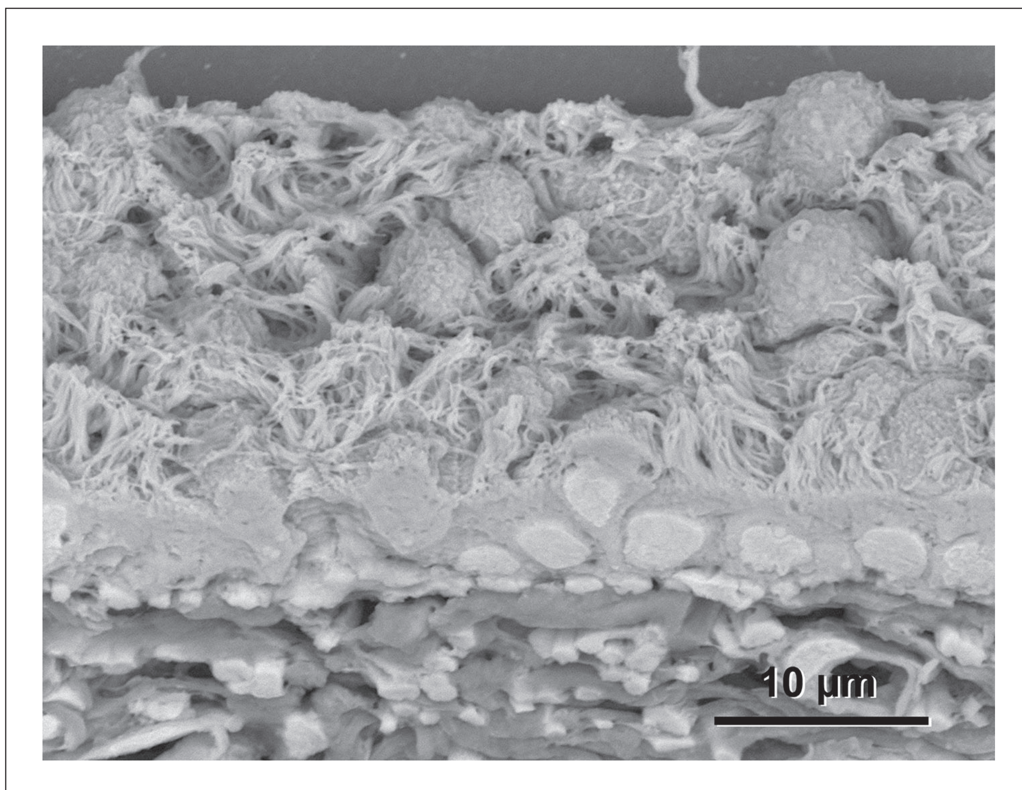

Fig. 7 Rat bronchioles fixed using 10% formalin solution (4% paraformaldehyde alone), which is typically used to fix histopathology specimens for light microscopy. The micrograph clearly shows the elaborate architecture of the ciliated epithelium (top) and collagen fibers with a crisscrossed, layered structure (bottom). Section thickness: 20 µm.

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6. Improving the Scientific Accuracy of Micrographs Using Correlative Light and Electron Microscopy (CLEM)

Correlative light and electron microscopy (CLEM) is a technique for improving the scientific accuracy of electron micrographs. CLEM combines optical and electron microscopy to produce images with electron-microscopy resolution so that instead of “missing the forest for the trees”, users are able to “observe the forest, select a tree, and then examine its leaves”. Figure 8 shows a rat renal corpuscle sample treated with our protocol and imaged with CLEM. To produce this image, a sample stained with hematoxylin and eosin was observed under a light microscope, and then the cover slip was removed to expose the sample, which was stained using potassium permanganate and lead and then observed using low-vacuum scanning electron microscopy. The protrusions of the podocytes seen with light microscopy (arrow) are imaged at a resolution not possible with light microscopy, allowing the fine-grained structure to be observed.

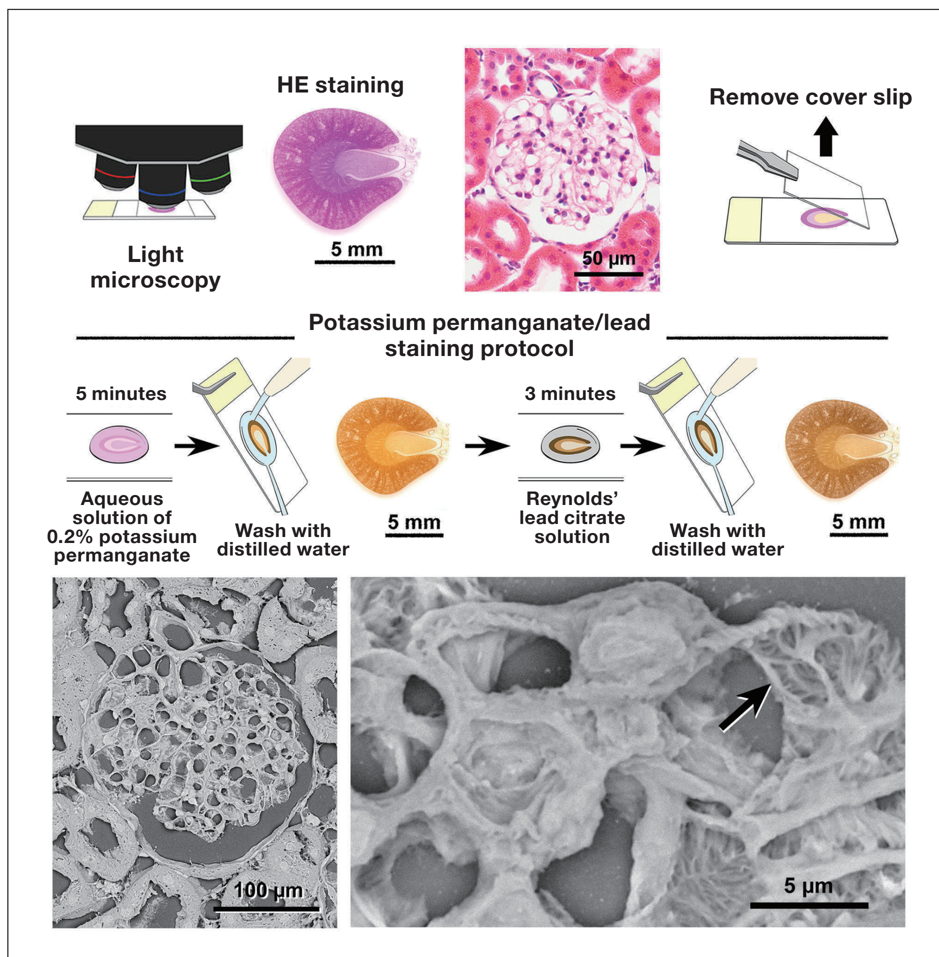

Fig. 8 Micrographs of rat renal corpuscle imaged with CLEM. The sample was first stained with hematoxylin and eosin and observed using light microscopy. The cover slip was then removed, and the sample was stained with potassium permanganate/lead and observed using low-vacuum scanning electron microscopy. The area observed with light microscopy is seen in elaborate detail. The protrusions of the podocytes seen with light microscopy (arrow) are imaged at a resolution not possible with light microscopy, revealing the fine-grained structure.

When the TM4000PlusII is used, real-time images of a section obtained by the CCD camera in the instrument show the area to be observed at electron-microscopy resolution. This handy standard feature greatly simplifies CLEM (Figure 9). With this tool, users can “observe a section, select a tissue region, and then examine its cells” just as in the “observe the forest, select a tree, and then examine its leaves” idiom mentioned above.

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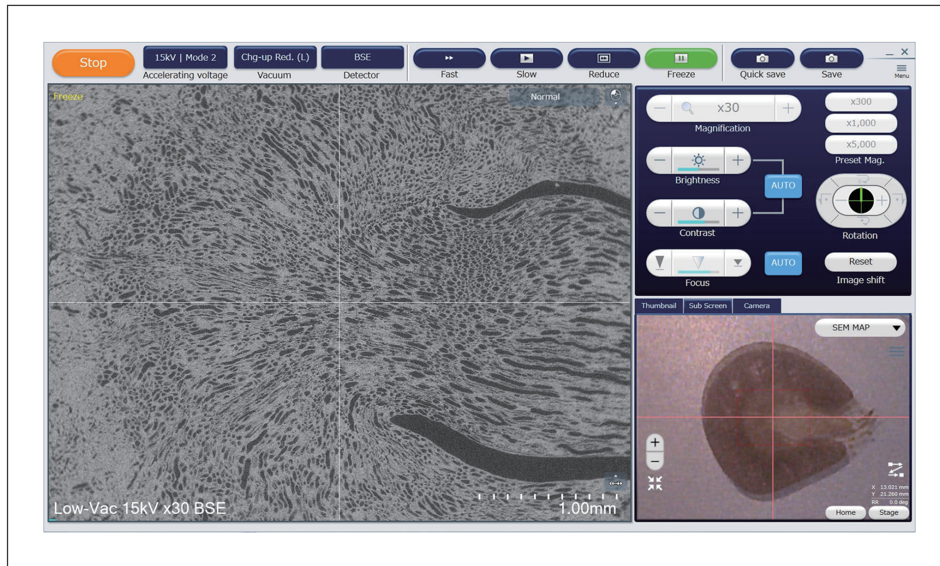

Fig. 9 Operating screen for TM4000PlusII. Real-time images of a section obtained by the CCD camera in the instrument (lower right) show the area to be observed at electron-microscopy resolution (left). This handy standard feature greatly simplifies CLEM.

7. Analyzing the Fine-grained Structure of Cultured Cells for Regenerative Medicine Research

Our protocol can be used to image, at electron-microscopy resolution, the three-dimensional structure of adhesive cells cultured on a light microscope slide. The micrographs of the SUIT-2 pancreatic cancer cell line shown in Figure 10 clearly show the intricate adhesions and connections between cells not visible at light-microscopy resolution.

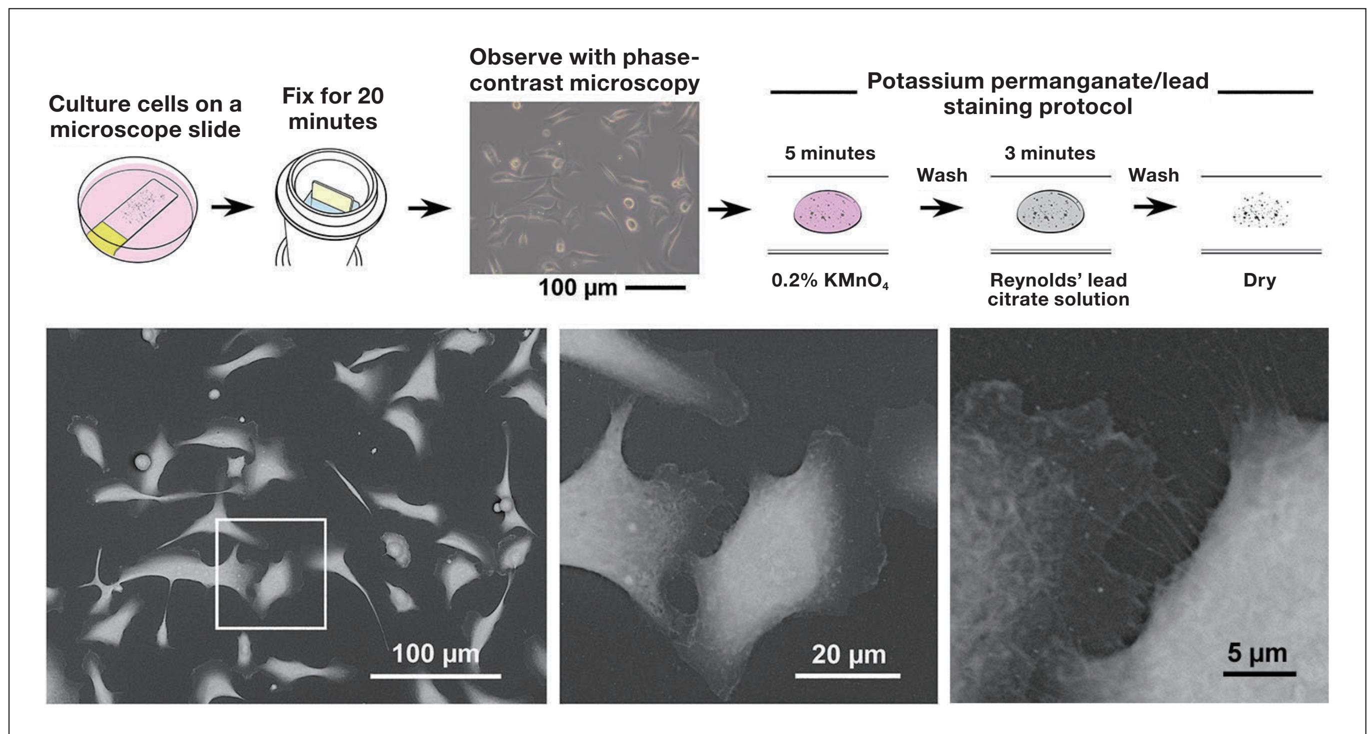

Fig. 10 Micrographs of cultured cells (SUIT-2 pancreatic cancer cell line). Once the best area for observation is selected using phase-contrast microscopy, potassium permanganate/lead staining is performed, and the specimen is observed using low-vacuum scanning electron microscopy. The micrographs show the intricate adhesions and connections between cells not visible at light-microscopy resolution.

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8. New Applications of Tabletop Low-vacuum Scanning Electron Microscopes to Medical Research and Clinical Diagnosis

This article has described a new electronic staining technique that does not require uranium compounds. As stated at the beginning of the article, these compounds have long been a problematic but necessary component in staining. With the proposed protocol for conveniently and rapidly imaging the detailed architecture of the cells and tissues that make up the organs of the body at electron-microscopy resolution, tabletop low-vacuum scanning electron microscopes are finding new biomedical and clinical applications such as the histopathological diagnosis of tissue and evaluation of the morphological quality of regenerated organs.

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全新无铀染色方法配合低真空扫描电镜将医学研究和临床诊断工作提升至新阶段
～开发了一种新型电子染色剂，将光学显微镜研究的石蜡切片样品转化为可用于SEM观察的切片～

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1. 前言

维持生命的每个器官，包括心脏，肾脏和肝脏，都具有由发挥着特定生理功能的细胞和结缔组织编织而成的精密三维结构。如果这种结构被破坏，生理功能就会受损，正常的生态就会转变为异常的病理状态，从而出现各种症状。病理组织学诊断通过形态变化来寻找这些变化的原因，主要使用光学显微镜进行，而研究细微的形态变化则需要电子显微镜。但由于电镜样品的制备方法复杂耗时，以及需要受到了严格管控的铀化合物进行电子染色，使得电子显微镜的应用受到了阻碍。此外，在再生器官的开发和研究中，三维结构的再现是一个迫切需要解决的课题，人们渴望开发一种简单快速的电子显微镜样品制备方法，能够以高分辨率捕捉培养细胞的微观结构和器官内部的三维结构，以及可用于再生器官质量评估和筛选的显微镜技术。

为了解决这些问题，我们一直致力于"无需铀化合物的新电子染色方法开发"这一长期课题，并最终成功建立了一种方案，可以在电子显微镜水平上以简单，快速的方式高分辨率捕捉构成器官的细胞和组织的微观形态。在本文中，我们将介绍台式低真空扫描电子显微镜新的应用方向，内容涵盖了病理组织诊断和再生器官形态质量评估等广泛的医学研究与临床应用等领域。

2. 使用便捷的载玻片专用holder和无荷电观察的低真空模式

台式扫描电子显微镜TM4000PlusII，顾名思义，是一款可放置于桌面的紧凑型扫描电镜，它配置了一个holder，可用于装载有石蜡切片的光学显微镜所用载玻片（图1）。该专用holder可实现载玻片的快速装卸，如果是厚度为5至30 μm的石蜡切片，则无需繁琐的高度调节。此外，拍摄的图像文件中会附带位置信息，每次重新放入载玻片的样品后，holder联通样品都可以调用已保存的位置信息，并自动找到保存好的观察位置。

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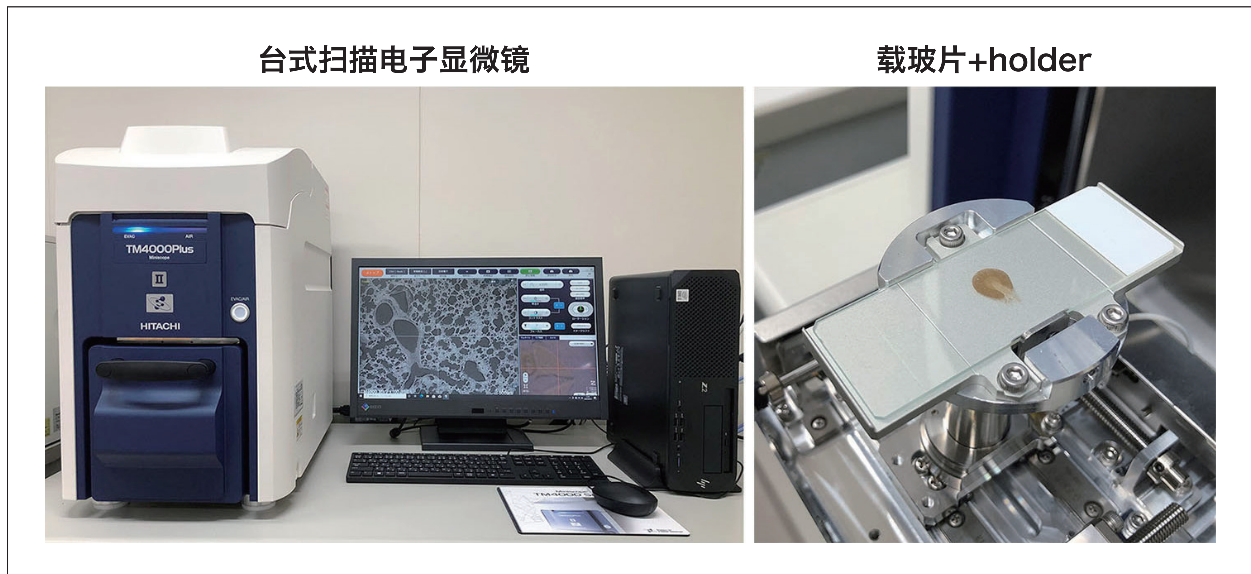

图1 （左图）台式扫描电子显微镜TM4000PlusII外观。（右图）专用的载玻片holder，装卸方便，无需进行高度调节。

低真空扫描电子显微镜可在30 ~ 50Pa的低真空度下工作，非导电性的石蜡切片和载玻片因入射电子而积聚的负电荷会被残留气体在低真空环境下产生的正离子中和，因此，可以进行无荷电的观察（图2）。

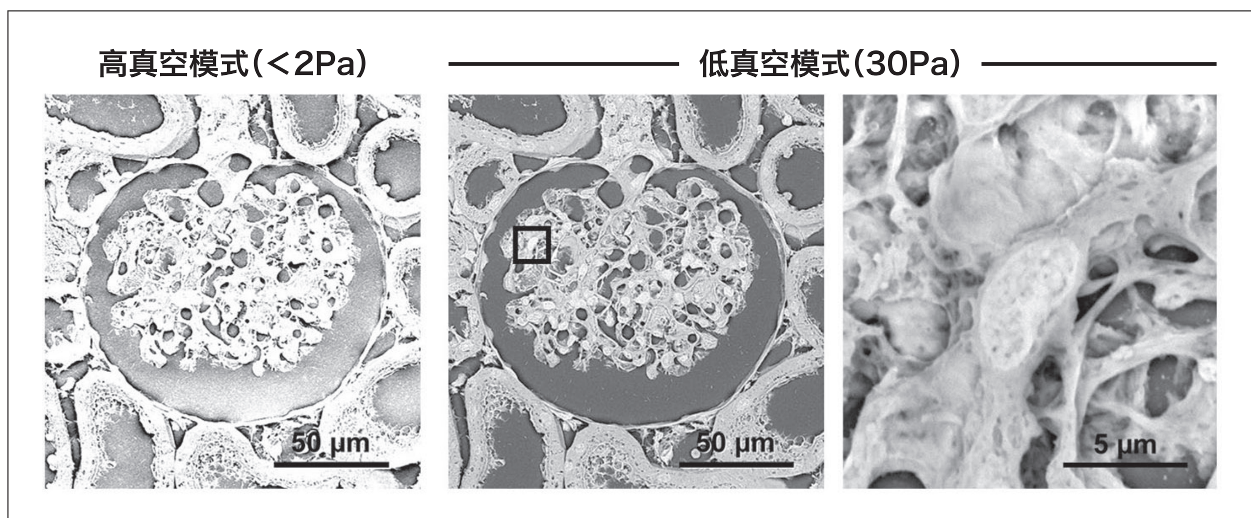

图2 高真空模式与低真空模式的图像对比。样品为大鼠的肾小球。（左图）高真空模式（<2Pa），荷电明显，无法进行正常的观察。（中图和右图）低真空模式（30Pa），荷电大幅减少，可以高倍率对中间照片中的方框内部分进行细节观察（右图）。

3. 将光学显微镜石蜡切片转化为电子显微镜可用样品的电子染色新方法开发

传统的电子染色方法是Watson于1958年开发的醋酸铀-铅染色法[1,2]，但铀化合物管控严格，从购买到储存、使用到废弃，处理都极其困难。因此，电子显微镜分析工作者们长期以来一直渴望一种不需要铀化合物的新型电子染色法。成功开发的“高锰酸钾-铅染色法”是一种简便而快速的方案[3]，它将光学显微镜用的石蜡切片用0.2% 高锰酸钾水溶液处理5分钟，并用水冲洗后，再用Reynold柠檬酸铅溶液处理3分钟，清洗干燥后可进行观察（图3）。与传统方法的唯一区别在于将2% 醋酸铀替换为0.2% 高锰酸钾，电子染色所需时间保持不变，约为10分钟。

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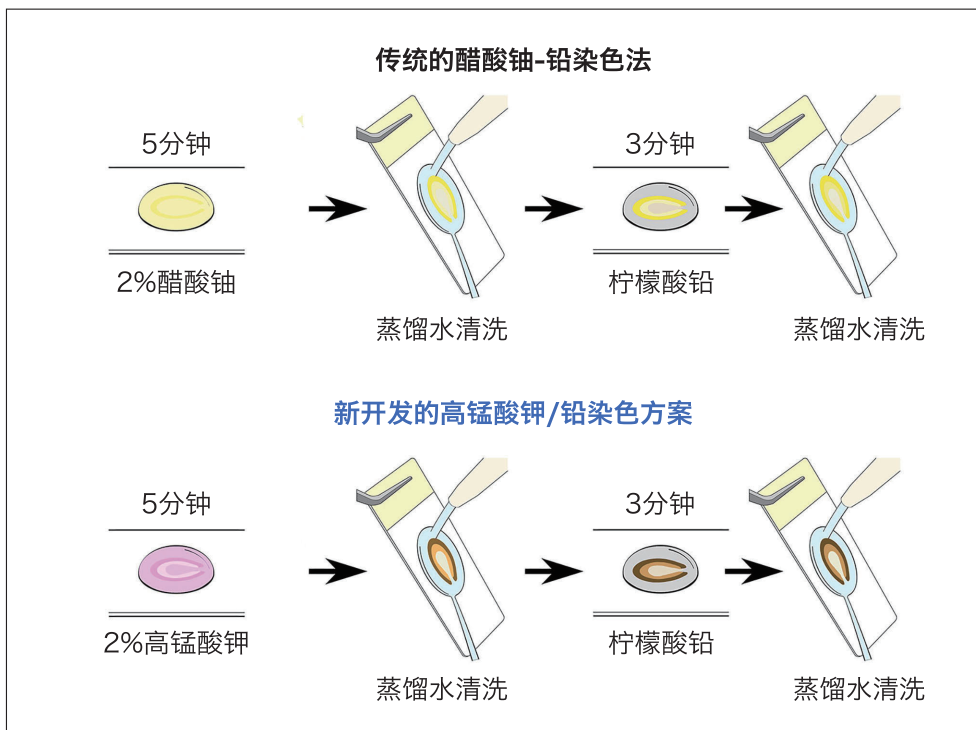

图3　传统的醋酸铀-铅染色法(上)和新开发的高锰酸钾-铅染色法(下)。

新的“高锰酸钾-铅染色法”可获得与传统的醋酸铀-铅染色法同等的高对比度染色结果，将光学显微镜制备的样品转化为电子显微镜样品的能力以及低真空扫描电子显微镜的强大功能是显而易见的。经过元素分析表明，高锰酸钾特有的氧化作用可提高铅的沉积，并且可以获得足够多的BSE电子，将细胞和组织的显微结构可视化表达[3]。

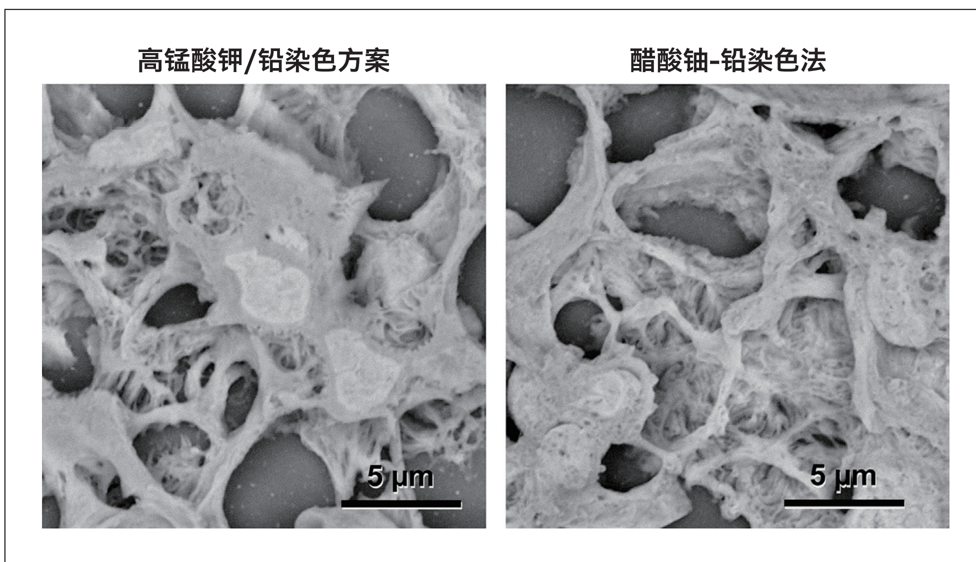

图4　大鼠肾小球。这种比较突出了将用于光学显微镜的样品转化为电子显微镜样品的能力的可靠性，以及低真空扫描电子显微镜的强大功能。固定剂＝2%多聚甲醛 + 2.5%戊二醛混合夜。切片厚度＝5 μm。

石蜡切片观察通常在BSE背散射模式下进行，但由于不同的加速电压会导致获得的图像信息会有差异，因此需要根据观察目的使用不同的加速电压。使用5至20 kV范围内的4种加速电压分别拍摄的图像如图5所示，值得注意的是，在低加速电压下，对比度降低但细胞表面形态清晰，而在高加速电压下，对比度上升但细胞表面形态变得不清晰。根据过去的经验，我们建议在低于500倍的低倍率下使用15至20 kV，旨在清晰显示整体图像，在500倍或更高的高倍率下使用5至10 kV，旨在显示细胞表面的显微形态。

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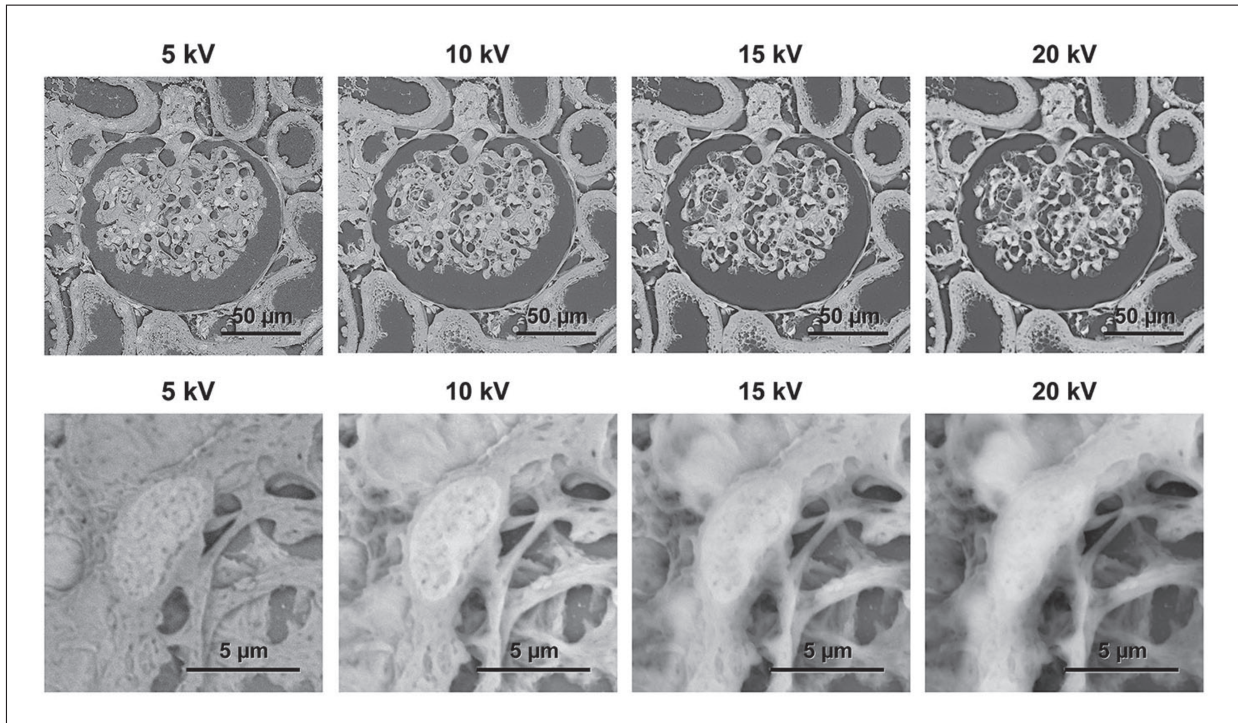

图5　比较不同加速电压下的图像信息。请注意，低加速电压会降低对比度，但细胞表面的形态清晰；高加速电压会提高对比度，但细胞表面形态不清晰。我们建议在低于500倍的低倍率下使用15-20 kV加速电压进行观察，在显示细胞表面的500倍或更高的高倍率下使用5-10 kV加速电压进行观察。

4. 使用厚石蜡切片观察法揭示细胞和组织形成的三维结构

通常，用于光学显微镜的石蜡切片被切成约5μm厚，以便于光线穿透，且细胞核在此厚度上不重叠。然而，我们之前报道了基于背散射电子检测的低真空扫描电子显微镜对厚石蜡切片（15 至 30 μm）[2] 的使用。更大的样品厚度允许对组织细胞的三维结构进行成像。图6显示了使用高锰酸钾-铅染色法观察到的大鼠肾小球、肾小管和纤毛细支气管表皮的实例。请注意，在所有情况下，在光学显微镜水平难以看到的微小纤毛等都可以通过台式扫描电子显微镜以三维方式捕捉到。

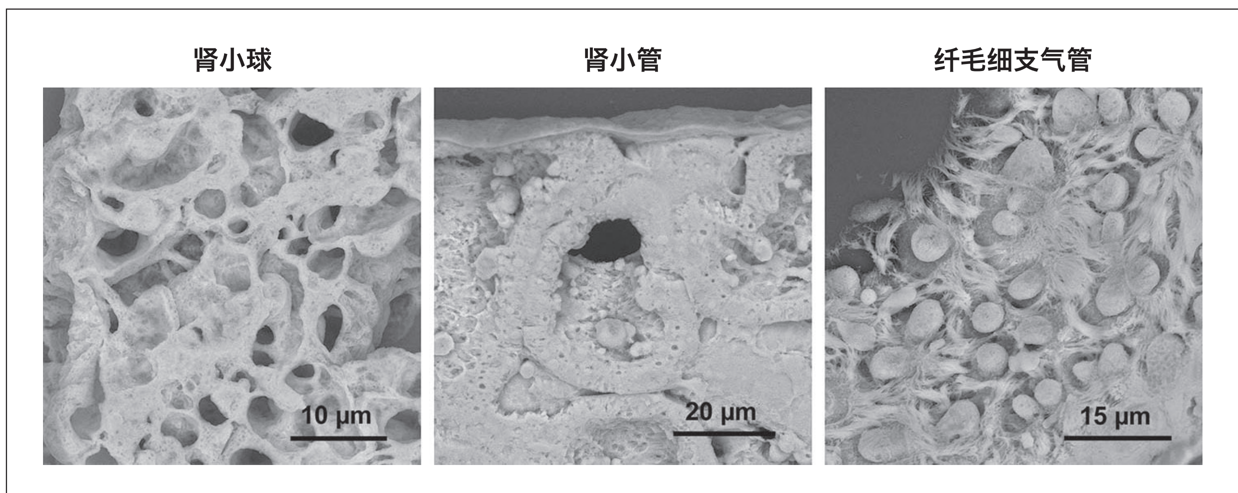

图6　厚石蜡切片观察法。将切片切成比通常的5μm更厚的20μm左右，深度更大，可清晰地看到由细胞编织而成的三维结构组织。大鼠器官。固定液＝2%多聚甲醛+2.5%戊二醛混合液。切片厚度＝20μm。

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5. 仅用福尔马林固定的组织病理学标本仍能以美丽，精致的细节成像

以上图像均为用制备电子显微镜样品的标准性半浓度Karnovsky固定液（= 2% 多聚甲醛和2.5% 戊二醛的混合溶液）固定的标本切片，但病理组织样品通常用10% 福尔马林溶液（仅4% 多聚甲醛）固定。图7显示了用10% 福尔马林溶液固定的大鼠细支气管的观察示例，从图中可以清晰地观察到纤毛表皮和呈现纵横层状结构的胶原纤维微细形态，表明本方法适合于观察病理组织标本。

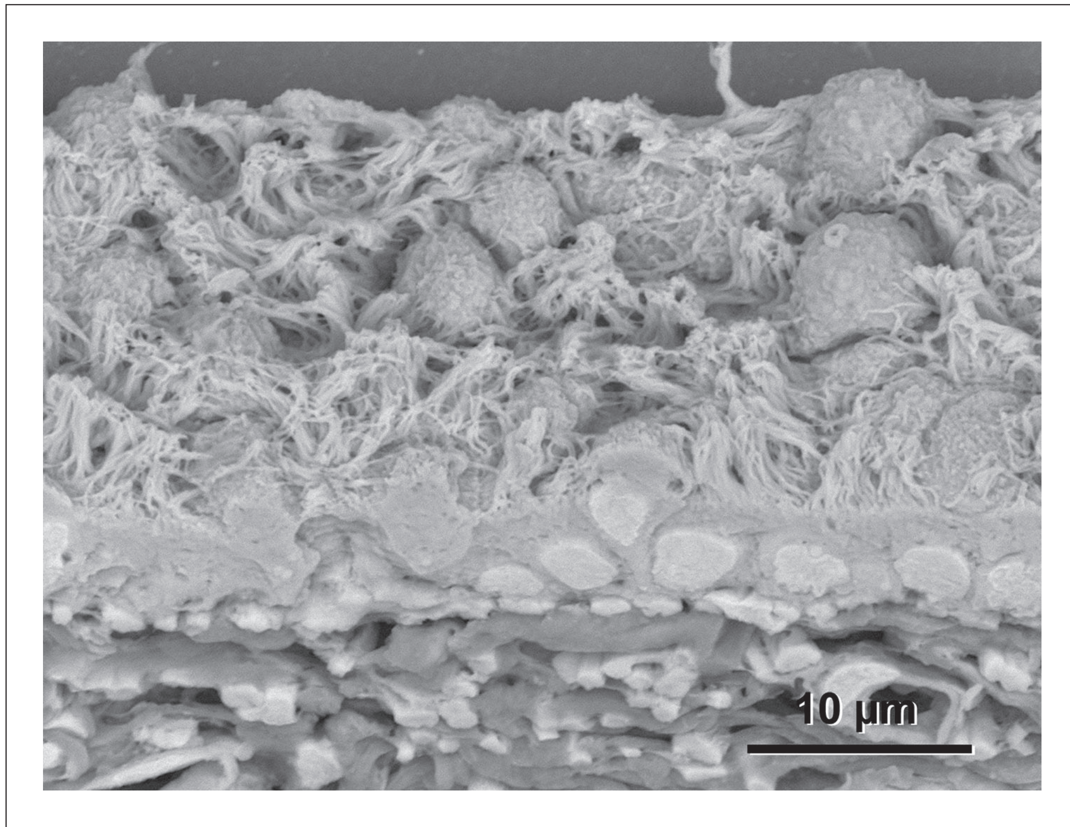

图7　经常用于光学显微镜病理组织标本的10%福尔马林溶液(仅含4%多聚甲醛)固定后的大鼠细支气管。纤毛表皮(上部)和呈现纵横层状结构的胶原纤维(下部)的微细形态清晰可见。切片厚度=20 µm。

6. 关联技术的应用：光学显微镜/电子显微镜关联技术(CLEM)的应用提高了观察图像的准确性

提高电子显微镜图像准确性的方法之一是“光学显微镜/电子显微镜关联观察法”（CLEM：Correlative Light and Electron Microscopy）。这是一种将光学显微镜拍摄的同一样品的显微位置，以电子显微镜水平高分辨率来显示的方法，是一种将“只见树木不见森林”改为“看到森林后选树并观察树叶”的方法。图8显示了使用该方法观察大鼠肾小球的示例。在光学显微镜下，用常规的苏木精-伊红染色进行观察后，取下盖玻片，实施高锰酸钾-铅染色，并使用低真空扫描电子显微镜观察后发现，光学显微镜拍摄的同一部位的足细胞突起（箭头所示）清晰可见，甚至包括在光学显微镜水平无法确认的细微结构。

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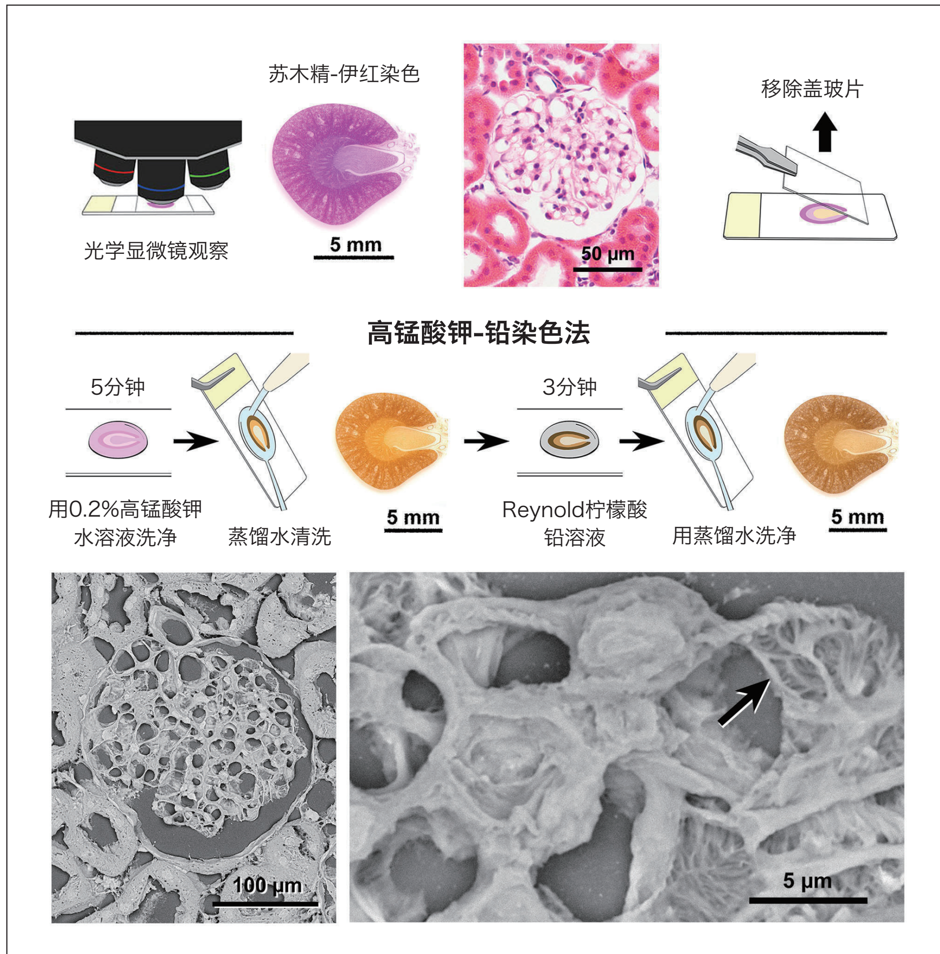

图8　使用光学显微镜-电子显微镜关联方法(CLEM)观察大鼠肾小球的示例。在采用常规的苏木精-伊红染色进行光学显微镜观察后，取下盖玻片，实施高锰酸钾-铅染色，然后在低真空扫描电子显微镜下进行观察。可以高分辨率观察用光学显微镜拍摄的同一部位，能观察到在光学显微镜水平无法确认的微细结构，并能识别足细胞的突起(箭头所示)。

如果使用TM4000PlusII，可以一边参考内置的CCD摄像头所显示的切片实时光学图像，一边在电子显微镜水平实时观察目标的显微位置，也就是说，其配备了一项便捷的功能，可大幅提高光学显微镜-电子显微镜关联工作的效率（图9）。正如“看到森林后选树并观察树叶”那样，它可以帮助用户“查看切片，选择组织，然后观察其细胞”。

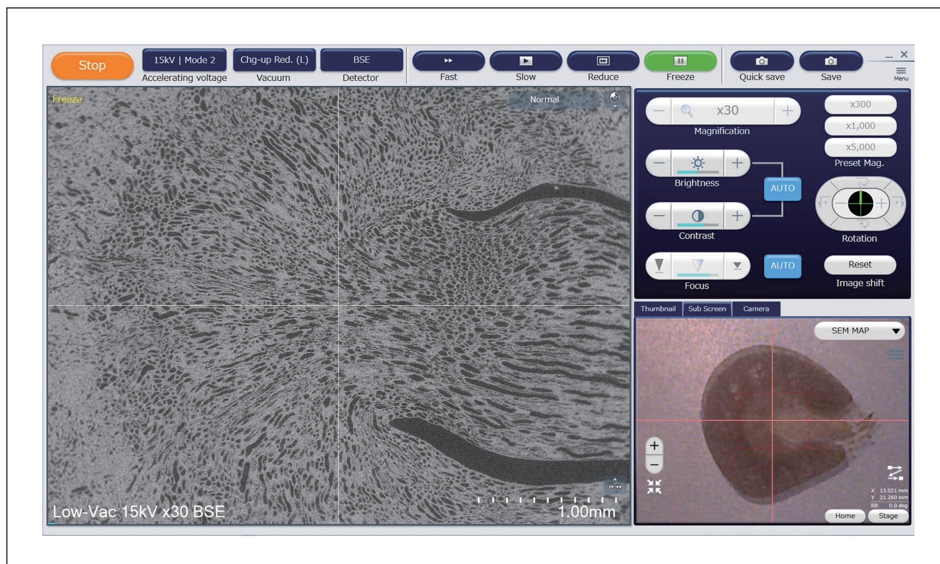

图9　TM4000PlusII的操作画面。标配了可大大提高光学显微镜-电子显微镜关联工作效率的便捷功能，可以一边参照内置CCD摄像头显示的切片实时影像(画面右下角)，一边在电子显微镜水平(画面左侧)对目标的观察部位进行实时观察。

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7. 可应用于再生医疗研究的培养细胞显微结构解析

通过应用本方法，可以在用于光学显微镜的载玻片表面培养具有粘附性的细胞，并在电子显微镜水平以高分辨率，将培养细胞的三维微观形态可视化。在图10所示的胰腺癌细胞株：SUIT-2的观察示例中，可以清晰地确认在光学显微镜水平无法捕捉到的细胞间微细粘附和联络形态。

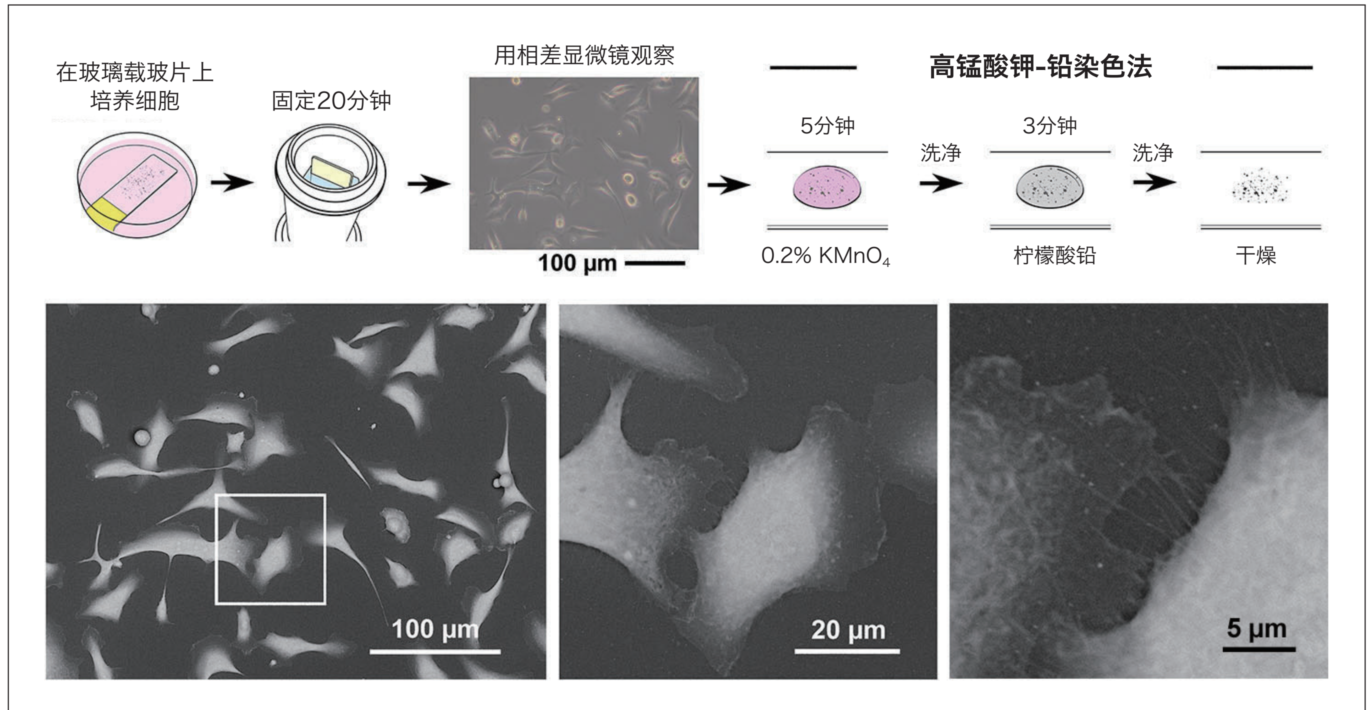

图10 培养细胞（胰腺癌细胞株：SUIT-2）的观察示例。使用相差显微镜选定最佳观察区域后，实施高锰酸钾-铅染色，并用低真空扫描电子显微镜进行观察。结果观察到了在光学显微镜水平无法捕捉到的细胞间微细粘附和联络形态。

8. 台式低真空扫描电子显微镜将医学研究和临床诊断提升到新阶段

本文介绍了文章开头提到的长期存在的问题，以及无需铀化合物的新型电子染色方法开发成功的概要。随着能够在电子显微镜水平以高分辨率方便而快速地捕捉构成生物器官的细胞和组织显微形态的方法确立，台式低真空扫描电子显微镜正在迈入一个新阶段，以期在病理组织诊断和再生器官的形态质量评估等广泛的医学研究和临床应用中取得成功。

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In Search of the Whereabouts of Dugongs in Japan

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1. Introduction

The dugong (*Dugong dugon*) is a marine mammal in the order Sirenia and family Dugongidae living in shallow tropical seas from the west coast of Africa to the Indo-West Pacific. Japan's Nansei Islands mark the northern extent of the range of dugongs. Adults grow to about 3 meters in length and weigh up to somewhat less than 500 kg. Dugongs feed exclusively on seagrasses growing in shallow waters (Figure 1). Manatees (Amazonian manatee, West Indian manatee, and West African manatee), another extant member in the order Sirenia, live in the Atlantic Ocean in areas that do not overlap with the range of dugongs. Dugongs are estimated to live up to 73 years. Females give birth after a 14.5-month gestation period. Births are thought to be separated by 3 to 7 years. Newborns nurse for 13.6 to 17.6 months and also consume seagrasses while they nurse[1).

Worldwide, the dugong is threatened and listed as vulnerable (VU) on the IUCN Red List of Threatened Species (https://nc.iucnredlist.org/redlist/amazing-species/dugong-dugon/pdfs/original/dugong-dugon.pdf). The Washington Convention on International Trade in Endangered Species limits the trade of derived products. In Japan, dugongs are protected, and hunting restrictions are applied under the national Act on Protection of Cultural Properties, Act on the Protection of Wildlife and Hunting, and Act on the Protection of Fishery Resources as well as the Okinawa Prefecture Ordinance for the Protection of Endangered Wildlife. A recent study found that the East Asian population of dugongs (in the seas around China and Taiwan and Japan's Nansei Islands), located on the east edge of their range, is at greatest risk of extinction[2)].

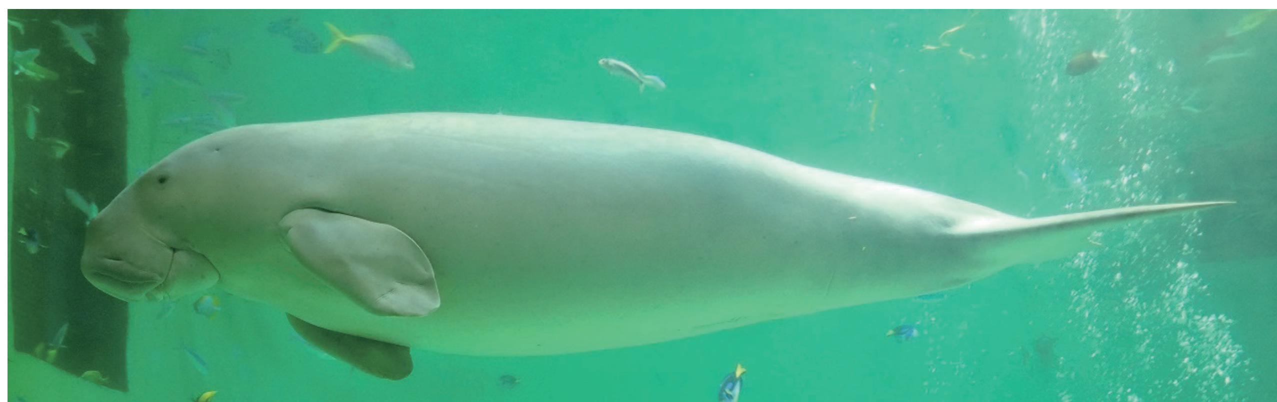

Fig. 1 A dugong swimming (Toba Aquarium)

With extreme hunting practices using dynamite beginning around the turn of the 20th century as documented in fishing records of Okinawa Prefecture and newspaper articles from the time, declines in dugongs in Japan appear to have pushed individual populations below the point of recovery[3)]. Nevertheless, intermittent sightings and bycatch of dugongs near Okinawa Island were reported beginning in the 1970s, and Japanese dugongs appear to have lived in small numbers around Okinawa until at least 2000. Moreover, interviews and submarine surveillance in the Sakishima

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Islands (the Yaeyama and Miyako Islands) around 2020 indicate that dugongs may still live in those areas. Somehow, the dugong population of the Nansei Islands miraculously and mysteriously survives, possibly helped by additions from migrants from the Philippines and other southern locations traveling in the Kuroshio Current[4]. Indeed, Okinawan and Philippine dugongs are very genetically close[5].

In efforts to protect Japanese dugongs, Japan's Ministry of the Environment and Okinawa Prefecture have been studying seagrass beds since 2001. (Okinawa Prefecture began doing this in 2016.) Before these studies, experts knew little about the distribution and ecology of Japanese dugongs. Bycatch and stranding information before the studies began suggested that dugongs lived only around Okinawa Island. Since then, however, dugongs have been found to live broadly throughout Okinawa Prefecture, and more information is now available about the seagrass beds that they feed from[6]. Projects launched to protect dugongs include ongoing efforts to increase awareness among fishermen of techniques for rescuing dugongs trapped in fishing nets to reduce bycatch accidents. Sightings tell researchers more about how the dugong lives, and fishermen have proven to be a valuable source of such information. Okinawa Prefecture has received about 550 reports of sightings to the present (via the Okinawa Dugong Portal Site of the Okinawa Prefecture Environment Department, Nature Conservation Division at https://biodiversity.okinawa/dugong/.) Sightings of dugongs and their feeding trails in Okinawa Prefecture since 2010 are shown in Figure 2[4].

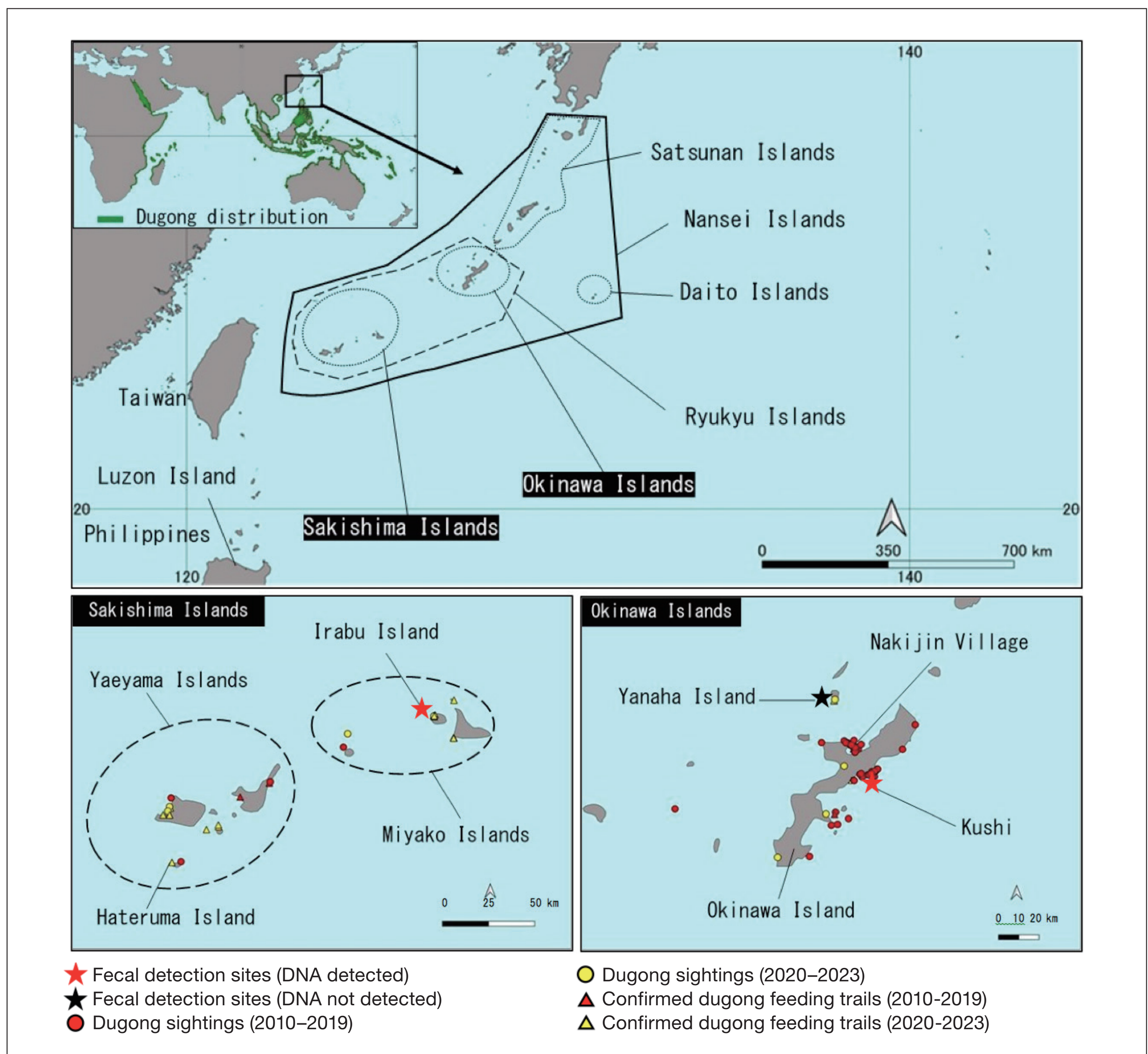

Fig. 2 Locations where feces of large herbivores were collected, animals thought to be dugongs were sighted, and dugong feeding trails were confirmed. The worldwide dugong distribution map at the top left is based on Marsh & Sobtzick, 2019[7].

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To this point, this paper has discussed the basic ecology of dugongs and efforts to protect them in Japan. The distribution of Japanese dugongs and their population sizes remain understudied. In 2022, we began checking for dugong DNA in the feces of large marine herbivores (e.g., dugongs, green sea turtles [*Chelonia mydas*]) very occasionally found in seagrass beds or washed up on shore. Previous studies had already estimated the distribution of dugongs based primarily on their feeding trails in seagrass beds (Figure 3), but we reasoned that the direct scientific evidence provided by DNA would elucidate the more recent distribution of this animal. Environmental DNA sampling for aquatic animals represents one approach for DNA analysis. This involves collecting water from areas where the target species lives or is raised and checking for DNA in these water samples to understand more about the animal's life or development. This technique is evolving rapidly in recent years, thanks in part to advances in analytical instruments.

Fig. 3 Feeding trails of dugongs observed in seagrass beds

With the assistance of the Toba Aquarium, we started developing a dugong primer in 2020 capable of specifically amplifying the DNA of this species. Our goal was to detect dugong DNA in seawater in the waters around Okinawa's islands using an environmental DNA approach[8]. We used the primer we developed to search for dugong DNA in seawater in the prefecture but, unfortunately, have yet to detect any. We are unsure exactly why but postulate that the extremely small amounts of dugong DNA present in the water mass and the fragile nature of DNA may have contributed. As we were working, a paper was published in 2021 on DNA extracted from the feces of dugongs and green sea turtles in Australia[9]. Although DNA has been previously sampled from the feces of terrestrial mammals, the approach of the investigators was astonishing in that they used feces of dugongs and green sea turtles collected from the marine environment. Reading the paper, we became convinced that DNA would be easier to detect in fresh feces than in the water mass and decided to use this approach to understand current dugong whereabouts.

Many readers may question why we do not just use aerial surveys to identify dugongs. Indeed, comprehensive and frequent surveys would provide population estimates and other information about Japanese dugongs. Okinawa, however, is surrounded by ocean and contains expansive tracts of seagrass beds along its coastal waters. A comprehensive survey would be prohibitively expensive. Moreover, failing to identify individuals in these minuscule populations would doom a survey, and while dugongs are easy to find in the open ocean, they are very hard to identify in shallow waters with coral sands. Cognizant of these difficulties, we narrow down the area to investigate based on sightings and other information, use drones to image seagrass beds, search for feeding trails and other traces of activity in the images, and then conduct underwater studies based on the findings.

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Following is a presentation of a 2024 paper on the identification of dugong DNA in feces collected in Okinawa Prefecture (Irabu Island and Okinawa Island) [4]. The study, which was initiated in 2022 and described in this paper, aimed to extract DNA from feces of apparent dugong origin collected in the wild with the goal of identifying new areas where dugongs live.

2. Dugong Feces

Feces presumably of dugong origin is sometimes found near seagrass beds and near the surf. Large clumps of feces very occasionally are found on the seabed near seagrass beds, but all feces we found off the shore of Irabu Island in 2022 in which dugong DNA was later detected was floating on the ocean's surface. The buoyancy of feces likely depends on how long ago it was excreted, what type of seagrass the animal ate, and the gastrointestinal health of the animal. Dugong feces resembles human feces in size and shape, is dark green, and smells remarkably similar to horse and cow feces. Some samples contain small amounts of undigested seagrass fiber (from underground stems and other parts). The feces of green sea turtles, who cohabit areas where dugongs live, is very similar to dugong feces. Green sea turtles also feed on seagrass, and the close similarity of the feces of the two species confounds the efforts of samplers to distinguish them. Not infrequently, large feces apparently from a dugong is revealed by DNA analysis to be from a green sea turtle. We were involved a television special called "In Pursuit of the Elusive Dugong in Okinawa"[10], which discussed dugongs off the shores of Irabu Island. The program highlighted the detection of dugong DNA in sampled feces. Many reports of feces sightings and samples have come from many places in the prefecture ever since.

3. Extracting and Analyzing Dugong DNA from Feces

3-1. From sampling to DNA extraction

Fecal samples collected on site must be stored in ethanol or another preserving medium and refrigerated as soon as possible to prevent DNA degradation. Ethanol, however, is rarely available to people living on Okinawa's remote islands to preserve the fecal samples they collect. They instead use the commercially available disinfectant Osvan (10% benzalkonium chloride solution by Alinamin Pharmaceutical), adding 0.5 mL per liter of seawater in which the sample is contained, and then freeze the sample. Now, storage containers, ethanol, and other materials are distributed to islands where fecal samples are often collected. Collected samples are quickly flown frozen to a laboratory on Okinawa Island for DNA extraction soon after arrival. Using the QIAamp Fast DNA Stool Mini Kit (Qiagen) about 100 mg is collected from the surface of the solid part of a fecal sample with a sterile spatula, with about 50 µL of liquid extract obtained. Fecal samples are normally rich in PCR-inhibiting substances, which must be removed with the InhibitEX Buffer in the kit.

3-2. PCR

We made a dugong-specific primer according to the methodology of the previous Australian study[9]. The primer sequences were F: 5'-CGCGCGCTATGTACTTCGT-3', R: 5'-GGGGTAAGTAGTGTAATGCACG-3', with a product size of 110 bp. Each sample of extracted DNA was placed in a well and subjected to 2-step PCR. The PCR solution contained 12.5 µL of iProof HF Master Mix (Bio-Rad), 0.5 µL portions of 10 µM primers, 1 µL of DNA solution, and 10.5 µL of sterilized water. The total volume was 25 µL. A Biometra Tone (Analytik Jena) was used as the PCR thermal cycler. PCR was performed by first holding the temperature at 98°C for 45 s and then performing 35 cycles each consisting of 10 s at 98°C, 30 s at 65°C, and 15 s at 72°C followed by an elongation reaction at 72°C for 5 minutes. Ultrapure water was used as a negative control. DNA extracted from the muscle of a dugong carcass found near the coast of the village of Nakijin, Okinawa in March 2019 was used as a positive control. The positive and negative controls were included in each PCR run.

Two-step PCR was performed to enhance the detection of DNA extracted from feces. This involved diluting the first-step PCR products by a factor of 100 with sterilized water and using the resulting solution as a PCR template in the second step. The primers and PCR conditions used in the second PCR step were identical to those used in the first.

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3-3. Electrophoresis and purification of amplification products

The amplification products of the second PCR step were visualized by electrophoresis. A Mupid-exU (Mupid) was used as the electrophoresis device. Agarose gel was prepared by combining 2 g of Agarose S Tablets (Nippon Gene), 6 µL of Midori Green Advance (Nippon Genetics) as a fluorescent DNA stain, and 100 mL of 1× TAE as the electrophoresis buffer liquid. 100 bp DNA Ladder (Takara Bio) was used as DNA molecular weight markers. An Illuminator UltraSlim UV (Gel Company) transilluminator was used to visualize amplification products. To obtain DNA for sequence analysis, products assumed to have a dugong-specific sequence (110 bp) were cut from the agarose gel. The DNA from the gel was processed as directed by the kit instructions using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). Concentrations of the extracted DNA were determined using the Quantus Fluorometer (Promega) and QuantiFluor dsDNA System (Promega).

Our analyses of feces collected from locations in the prefecture including Irabu Island and Kushi on Okinawa Island produced amplification products apparently of dugong origin (Figure 4) [4).

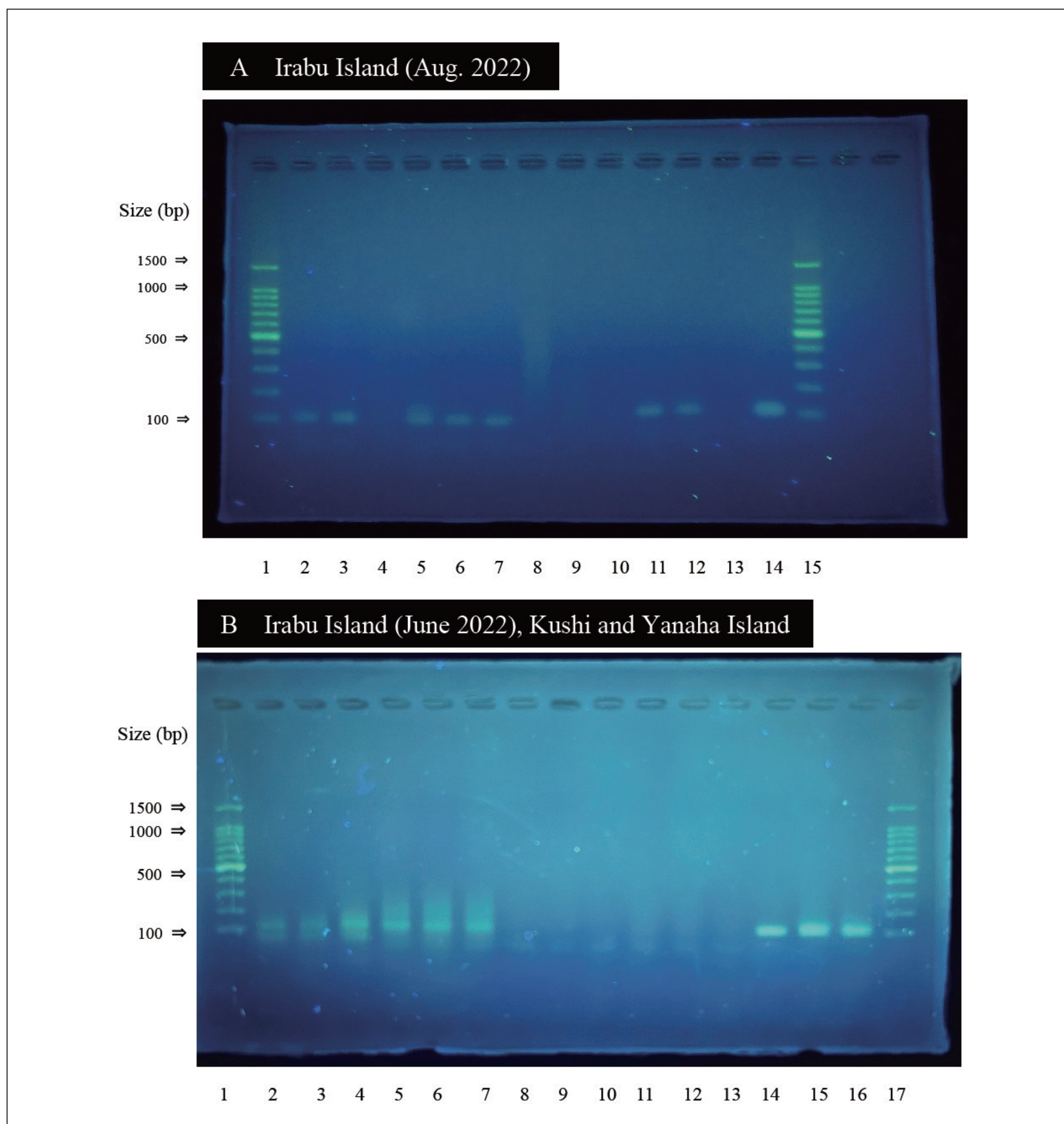

Fig. 4 Photographs of agarose gel electrophoresis patterns of amplicons obtained from PCR of feces using dugong-specific primers. Lanes A1 and A15: DNA ladders, A2-12: from a fecal sample collected from Irabu Island in August 2022, A13: negative control, A14: positive control, B1 and B17: DNA ladders, B2-4: from a fecal sample collected from Irabu Island in June 2022, B5-7: from a fecal sample collected from Kushi in the city of Nago, Okinawa, B8-10: from a fecal sample collected from Yanaha Island, B11-13: negative controls, B14-16: positive controls. Each lane represents a replicate of extracted DNA from a fecal sample. DNA was extracted from the fecal samples shown in Figure 4A as a single replicate and from the samples shown in Figure 4B in replicates of three.

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3-4. Nucleotide sequencing and database comparison

The sequences of the amplification products were determined and then compared against sequences in BLAST (National Center for Biotechnology Information). DNA was fluorescence labeled using the SupreDye v3.1 Cycle Sequencing Kit (EdgeBioSystems). Post-cycle sequence purification was done with the SupreDye XT Purification Kit (EdgeBioSystems). DNA sequence analysis was performed with the DS3000 compact capillary electrophoresis sequencer (Hitachi High-Tech). The sequences determined were compared against dugong base sequence information in BLAST (Figure 5). The sequences of the amplification products completely matched the sequence of dugong DNA registered in BLAST, demonstrating that dugongs still live in the waters off Irabu Island and Kushi on Okinawa Island.

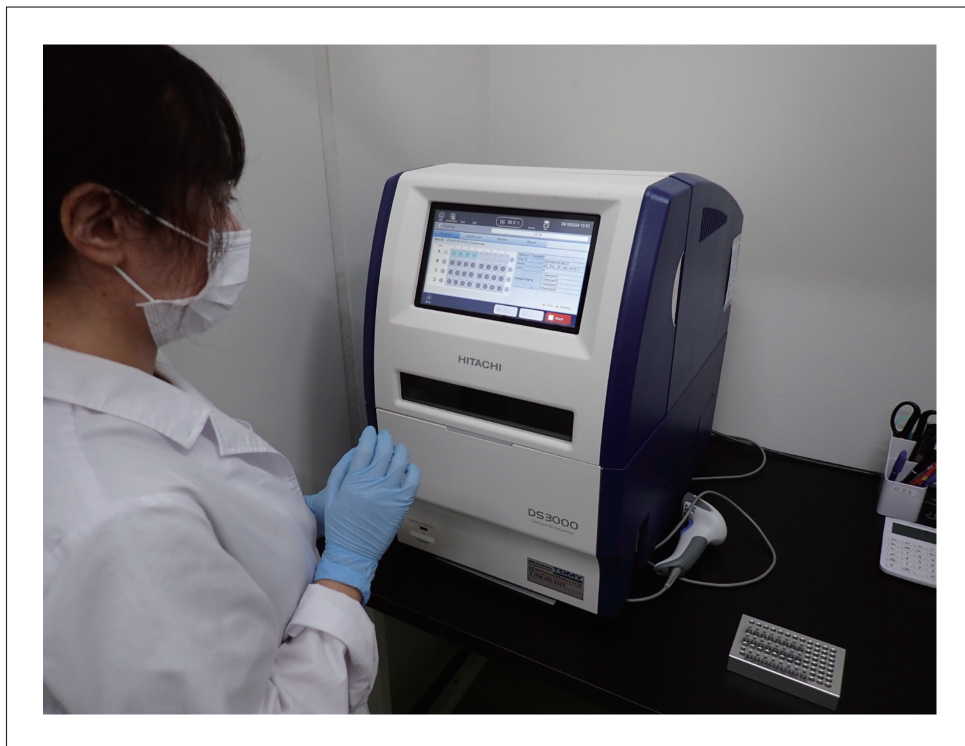

Fig. 5 An operator performs DNA sequence analysis on the DS3000 compact capillary electrophoresis sequencer (Hitachi High-Tech).

4. Conclusions

The dugong is an umbrella species indicative of biodiversity in tropical seagrass zones. Dugong extinction would lead directly to the degradation of shoreline ecosystems. The fact that a species of this size has managed to survive along the shores of islands in Okinawa is nothing short of a miracle and speaks of the richness of coastal nature there. As we seek to reach the goal of securing a nature-positive world, our generation should shoulder the task of preserving coastal ecosystems, including dugongs and the seagrass beds that nourish them.

Fecal analysis has added scientific evidence of dugong existence, which has until now only been speculated. We will proceed by attempting to use a recently published method for extracting nuclear DNA from feces[11] to distinguish individuals and obtain head counts of the still mysterious Japanese dugong subpopulation and also better characterize inter-island migration and other behaviors.

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Application of Rapid Bedside Therapeutic Drug Monitoring to Manage Sepsis With a Focus on Beta-lactam Antibiotics and Linezolid

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1. Introduction: The Importance of Optimizing Antibiotic Therapy in Sepsis Management

Sepsis is defined as "a condition in which a dysregulated host response to an infection leads to serious organ dysfunction[1]." Sepsis represents the most severe form of infection, with 20% to 30% of cases being fatal. Relevant Japanese and international guidelines stress appropriate early intervention as the key to improving outcomes, and one intervention is the early administration of appropriate antibiotics[1,2].

Conventional approaches for selecting appropriate antibiotics have focused on rapidly administering an antibiotic effective against the causative microorganism. More recent approaches, however, also emphasize optimizing the dose regimen by determining how much drug should be administered and at what dosing interval. Studies are producing a growing body of evidence about the continuous infusion and extended infusion of beta-lactam antibiotics frequently used in sepsis management, as well as therapeutic drug monitoring (TDM). This article presents the theoretical underpinnings of and current evidence supporting these approaches and also discusses the use of rapid bedside TDM in ICUs, which has recently become more prevalent.

2. Pharmacokinetic Changes in Patients with Sepsis: Increased Volume of Distribution and Augmented Clearance

Patients with sepsis often have blood antibiotic concentrations outside the appropriate window because of substantial changes in pharmacokinetics caused by the following physiological alterations[3].

(1) Increased volume of distribution: Sepsis causes increased vascular permeability, leading to extravasation of fluids. High-volume fluid resuscitation also increases effective circulating volume. These changes greatly increase the volume of distribution of water-soluble antibiotics, lowering their concentrations in the blood. Many critically ill patients have hypoalbuminemia. In those with hypoalbuminemia, highly protein-bound antibiotics occur less as protein bound and more in the unbound form. More drug moves to the tissues, increasing the volume of distribution as a result.

(2) Augmented renal clearance: Sepsis-associated inflammation, high-volume fluids, and catecholamine administration can increase renal blood flow, pushing renal function beyond normal levels. This condition is known as augmented renal clearance (ARC) and is typically defined by a 24-hour creatinine clearance (CrCl) of greater than 130 mL/min/1.73 m^2. Young males and those with trauma/head trauma, burns, or sepsis are at increased risk of ARC. In a meta-analysis of critically ill adults, ARC was seen in 39% of the overall population and 33% of those with sepsis[4]. Those with ARC experience the early elimination of renally excreted drugs and have difficulty achieving target concentrations at standard doses.

(3) Decreased clearance from organ dysfunction: Sepsis frequently causes acute kidney injury, and this results

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in a condition opposite to ARC. Delayed drug elimination due to decreased renal function leads to overdosage at standard doses, which in turn increases the risk of adverse events due to elevated blood concentrations (e.g., neurological, hepatic, and renal adverse events due to beta-lactam antibiotics). Patients with this condition are often on extracorporeal membrane oxygenation (ECMO) or continuous hemodiafiltration (CHDF). The changes these cause in drug absorption and removal and volume of distribution reduce blood concentrations. Blood concentrations become even more difficult to predict as a result.

The takeaway points are that there is substantial variation in pharmacokinetics among individual patients with sepsis, and that some patients have insufficient (or excessive) levels when treated at normal antibiotic doses.

3. Theoretical Underpinnings of and Evidence Supporting Continuous Infusion of Beta-Lactam Antibiotics

Beta-lactam antibiotics are a class of agents characterized by time-dependent antimicrobial activity. Their efficacy is therefore highly dependent on the proportion of time during which their blood concentration exceeds the minimum inhibitory concentration (MIC) of the responsible microorganism (%T>MIC): the longer this time, the greater their antimicrobial activity. A %T>MIC of 40% to 70% is a typical target in general infection management. Given that critically ill patients require higher concentrations, the European Society of Intensive Care Medicine recommends a %T>MIC of 100% in a position paper[5)]. One guideline recommends continuously exceeding the level at 4 times MIC (100% fT>4×MIC) [6)].

In practice, however, these targets are often not achieved. In an international multicenter study of beta-lactam antibiotic blood concentrations in patients in the intensive care unit (ICU) for sepsis and other conditions, the target 100%fT>MIC was not achieved in almost 40% of the patients[7)]. Insufficient antibiotic levels should be avoided because they lead to treatment failure and the development of resistant strains. Sufficient antibiotic levels must therefore be maintained in critically ill patients in particular.

Continuous infusion and extended infusion are two approaches based on the pharmacokinetic/pharmacodynamic (PK/PD) characteristics of beta-lactam antibiotics for achieving target levels. For example, infusing meropenem or piperacillin/tazobactam continuously or as an extended infusion (of at least 3 hours) produces concentrations above MIC with fewer troughs than when these drugs are given as intermittent infusions of 30 minutes to 1 hour (Figure 1). Continuous infusion keeps concentrations above MIC throughout the day, which is particularly beneficial for patients with ARC or otherwise augmented renal clearance, critically ill patients, and patients infected with a microorganism with a high MIC. Continuous and extended infusions are now widely used in American and European ICUs. In an international, multicenter survey whose results were published in 2023, beta-lactam antibiotics, when used, were given as an extended or continuous infusion in about 60% of ICUs[8)].

Evidence supporting the continuous infusion of beta-lactam antibiotics is growing. Suffering from small sample sizes, the studies conducted through 2023 failed to sufficiently demonstrate efficacy. However, in 2024, the results of an international, multicenter, randomized, controlled trial (RCT) showing the benefit of continuous meropenem and piperacillin/tazobactam infusions in 7200 patients with sepsis (the BLING III trial) were announced[9)]. No significant difference in mortality at day 28 was shown between the patients given continuous and intermittent infusions, whereas the former population achieved better improvement in clinical symptoms. A 2024 meta-analysis of 18 RCTs including BLING III (total of 9108 patients) found significantly lower mortality at day 90 associated with continuous/extended infusion (25.7% for extended vs. 28.2% for intermittent, relative risk: 0.86, 95% CI 0.72-0.98), as well as improved clinical cure rates[10)]. Such evidence has demonstrated the benefit of continuous infusion of beta-lactam antibiotics for sepsis to an extent to which this therapy is weakly recommended in Japanese and international guidelines on sepsis management[1,2)].

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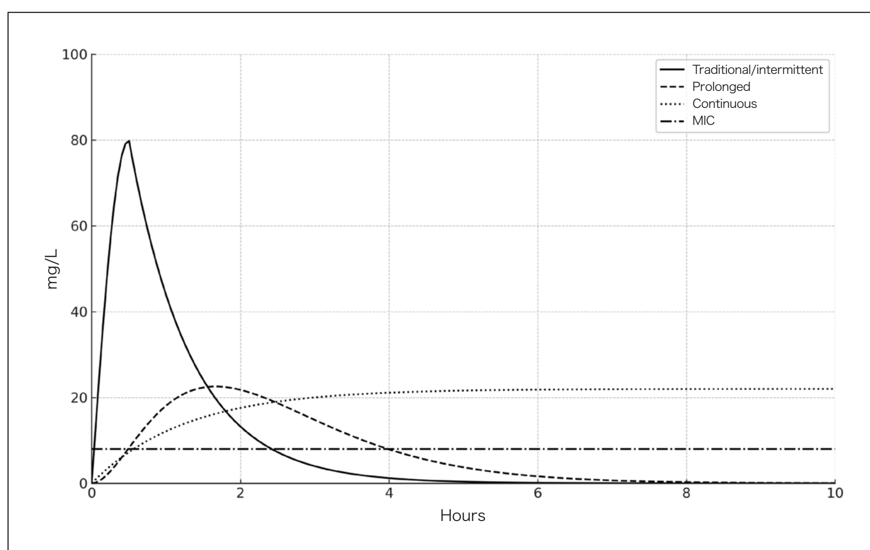

Fig. 1 Changes in blood concentrations of beta-lactam antibiotics given with different infusion approaches

4. The Need for and Benefit of Therapeutic Drug Monitoring for Beta-lactam Antibiotics

Maintaining sufficient blood levels of antibiotics in patients with sepsis is key to treatment success, even though adequate levels are often not achieved in practice. Developing treatment strategies such as continuous infusions may improve exposure, but whether a patient has achieved an appropriate blood concentration is still unclear. Beta-lactam antibiotics are generally well tolerated, but they can be hazardous at extremely elevated blood concentrations. One study found that the risk of neurotoxicity increased to 50% at trough concentrations exceeding 360 µg/mL of piperacillin and 64 µg/mL of meropenem[11]. Though the adverse events reported in the continuous infusion arm of the BLING III trial did not differ significantly from those in the intermittent infusion arm, serious central neurological adverse events were reported in the continuous infusion arm, and elevated blood concentrations may have been involved. Blood concentrations must be appropriately managed to both ensure therapeutic efficacy and avoid adverse events. For this, TDM is a solution for measuring blood concentrations and adjusting doses.

In TDM, the blood concentration of a drug is measured, with the results used to adjust the dose or dosing interval. TDM is performed to optimize the efficacy and safety of pharmacotherapy in patients and is used mainly with drugs having a narrow therapeutic window or large inter-individual variation in pharmacokinetics. Beta-lactam antibiotics were not the subject of routine TDM given their relatively wide safety margin, but more experts are calling for TDM for beta-lactam antibiotics to be used in the management of severe infections.

Data suggest that using TDM may improve clinical outcomes in severe infections. In a multicenter RCT, Hagel *et al.* compared TDM with conventional practice in 249 patients with sepsis treated with continuous infusions of beta-lactam antibiotics (piperacillin/tazobactam). Significantly more of those managed with TDM achieved target concentrations than those managed without TDM (37.3% vs. 14.6%)[12]. A meta-analysis of 8 studies that included this trial concluded that TDM might reduce treatment failure, although the heterogeneity of the studies was high[13]. The Japanese Clinical Practice Guidelines for Management of Sepsis and Septic Shock 2024 weakly recommends (Grade 2D) “Administering antibiotic treatment with TDM in sepsis.”[1] These developments show the increasingly prominent role of TDM in sepsis management. More institutions are adding TDM to their treatment protocols. The above-mentioned, international, multicenter study found that about 40% of responding institutions used TDM for beta-lactam antibiotics, and that the proportion exceeded 50% in institutions in Europe and Central and South America[8]. As these highlights of the above studies suggest, TDM for beta-lactam antibiotics is attracting attention as an effective tool for better managing sepsis and preventing antimicrobial resistance.

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5. New Developments in TDM for Linezolid

A growing body of evidence supports TDM for the anti-MRSA drug linezolid in addition to beta-lactam antibiotics. Linezolid is commonly used to treat severe infections such as ventilator-associated infections, but it can cause myelosuppression (e.g., thrombocytopenia, anemia) as an adverse reaction. The likelihood of linezolid causing myelosuppression grows with both prolonged administration and elevated blood concentrations. A study found that blood trough concentrations exceeding 7 μg/mL increase the incidence of thrombocytopenia and proposed a target blood concentration of 2 to 7 μg/mL to achieve therapeutic efficacy while avoiding adverse events[7)]. Since linezolid is typically given at a predefined dose regimen irrespective of renal function, overdoses can occur in patients with certain background factors. Advanced age and renal failure are known to increase the likelihood of elevated blood concentrations. In a study involving patients aged 65 years or older, over half of the patients had trough concentrations exceeding the target range[14)]. In my practice, I have encountered patients with sepsis on blood purification therapy due to renal failure who have quite high blood concentrations after linezolid administration and suffer myelosuppression in the early stages of treatment.

The findings of a meta-analysis suggest that TDM could prevent serious adverse reactions[15)]. There have been recent attempts to use TDM for linezolid to adjust the dosage to individual patients and thereby reduce the risk of adverse reactions.

6. Issues in TDM Adoption and Rapid TDM with the LM1010 to Resolve These Issues

As mentioned above, despite its theoretical benefits, TDM for beta-lactam antibiotics and linezolid has yet to be widely adopted in Japan. Several hurdles are responsible (Table 1). The largest of them are a lack of evidence involving hard outcomes such as mortality and the previous absence of instruments that allowed convenient measurements.

Table 1 Major barriers impeding the adoption of TDM for beta-lactam antibiotics (created by the author in reference to Reference 16)

Type	Examples of barriers	Solutions
Knowledge	• Lack of data on clinical outcomes • Lack of understanding by physicians of target concentrations • Lack of cooperation between infection teams and intensivists • Variability in indication criteria for TDM • Lack of consensus on optimal PK/PD targets • Lack of cost-benefit data • The existing guidelines do not highlight the need for dose optimization	• Accumulate PK data and outcome data (outcome improvement and prevention of resistant strains) from critically ill patients • Obtain regular advice about treatment design from pharmacists and other experts and enhance cooperation among experts (intensivists, infectious disease specialists, microbiological laboratory technicians, pharmacists) • Identify patient populations in which use of TDM will be effective, such as patients with ARC • Present recommendations in guidelines
Access	• Little TDM infrastructure • Results take time • MIC information needed to set blood concentration targets is often unclear	• Develop instruments capable of analyzing frequently used beta-lactam antibiotics • Chromatography assays demonstrated to be adequate • Integrate measurement results into electronic medical records
Assays	• Complicated analytical technology is necessary (HPLC-UV, LC-MS/MS) • Assays take time	• Make once daily measurements routine • Develop rapid, convenient measurement procedures
Resources	• Analytical instruments and human resources are expensive	• Involve related people in workflow building • Develop implementation guidelines

The apparent benefits of TDM in theory contrasts with the lack of evidence in clinical practice. The study of Hagel *et al*. cited earlier found that the TDM arm achieved target blood concentrations significantly more than the control arm, but there was no significant difference in 28-day mortality (21.6% vs. 25.8%)[12)]. A meta-analysis that included the study of Hagel *et al*. and three other RCTs together with seven observational studies concluded that TDM reduced clinical symptoms and improved biological cure rates, but it did not significantly lower mortality[17)]. In a subsequent Dutch, multicenter RCT in patients admitted to the intensive care unit (DOLPHIN trial), neither mortality nor ICU length of stay differed between patients treated with TDM and those treated with standard dosing[18)].

There are several potential reasons for the lack of valid evidence involving hard outcomes such as mortality. First, previous studies were not limited to populations that would most likely benefit from TDM. Moreover, it takes time to initiate TDM after antibiotic treatment has begun. Finally, no studies with sufficient statistical power have been

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conducted. The RCT of Hagel *et al*., for example, had a high target blood concentration (4 times MIC), yet it targeted mostly susceptible causative microorganisms. The patients may therefore have achieved 100% fT>MIC even without TDM. The sample size of this RCT allowed the detection of a difference in the primary endpoint (SOFA score, or severity score), but the statistical power was likely insufficient to evaluate mortality. Of the diverse array of drugs used in the DOLPHIN trial, the most common was ceftriaxone, which has a long half-life. Moreover, the median time from the start of treatment to initial intervention was long (20 hours). As a result, target concentration achievement was comparable between the TDM and standard dosing arms. Reflecting on these studies, I am convinced that it would be more efficient to limit the use of TDM for beta-lactam antibiotics to specific populations that would most likely respond to continuous infusions, rather than to all patients with sepsis. Specific examples of populations for whom TDM should theoretically be indicated include: (1) critically ill patients with ARC or on CHDF or ECMO, who exhibit substantial variability in pharmacokinetics; (2) patients with burns or obesity; (3) patients infected with a high-MIC pathogen; and (4) patients on drugs such as meropenem or piperacillin/tazobactam that have a short half-life and are frequently used in sepsis management[19]. More evidence, however, is needed to verify these indications.

Another major barrier to implementing TDM is the need for proper equipment and technical expertise in general. Conventionally, measuring blood concentrations of beta-lactam antibiotics has required chromatography and been a complex, time-consuming process. The recently developed LM1010 high-performance liquid chromatograph may offer a solution. The LM1010 is a sophisticated analytical instrument that is designed to be easy to operate. Users can operate the instrument with no special technical expertise. With the LM1010, blood concentrations of meropenem, piperacillin, linezolid, and other antibiotics frequently used in intensive care settings can be quickly determined in-house. Using this instrument could break down many of the existing barriers and help generate evidence. We have an LM1010 in the ICU of our hospital that ICU-based pharmacists and intensivists use to determine blood concentrations in real time (Figure 2).

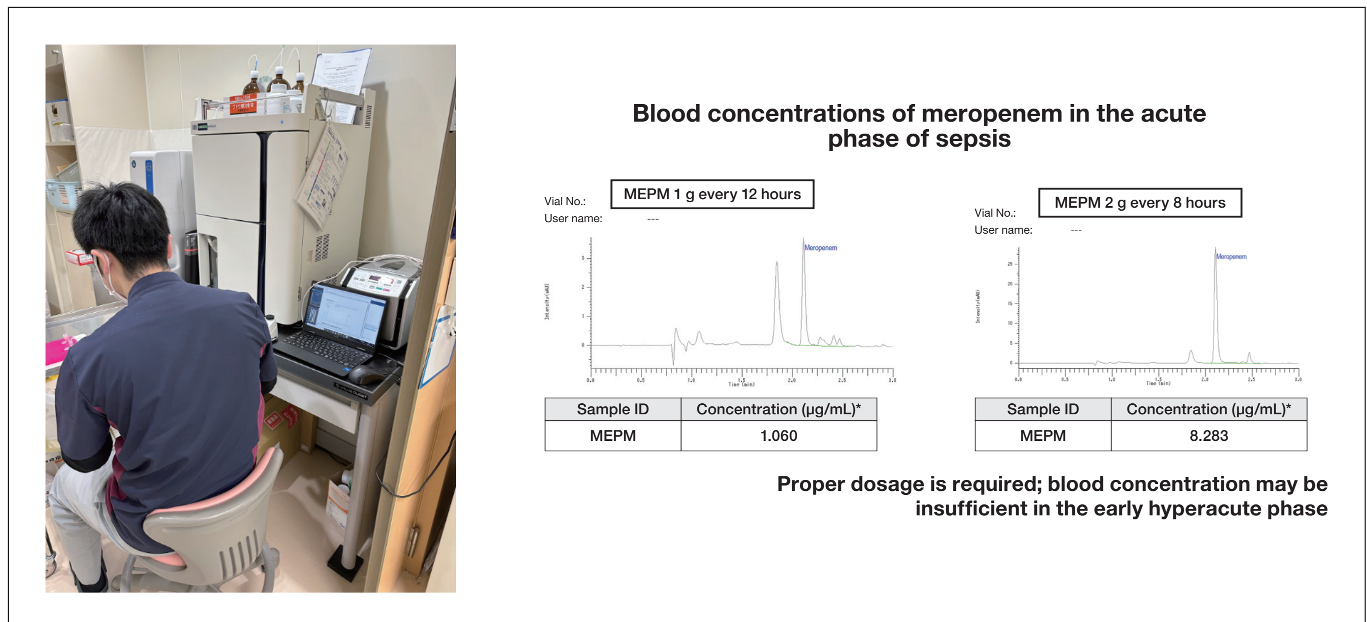

Fig. 2 Using the LM1010 for blood concentration analysis

7. Conclusions

Providing proper antibiotic therapy is key to improving the outcomes of patients with sepsis, and TDM is an important tool for achieving this goal. Recently developed analytical instruments and accumulation of knowledge are making quick implementation of TDM for drugs such as beta-lactam antibiotics and linezolid possible at the bedside. Although cost-related and other issues remain for implementation, the wider use of TDM promises to further improve the quality of sepsis management and lead to better outcomes for patients.

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A Novel Liquid Sample Observation Technique with the "Vitro Detector"

Mai Yoshihara*1, Mitsuhiro Nakamura*2

1. Introduction

In conventional scanning-electron microscopy (SEM), a sample for observation is placed inside a specimen chamber, which is evacuated to a state of high vacuum. The sample surface is scanned by an electron beam, and an image is acquired by detecting secondary electrons, backscattered electrons, or other signals induced by the irradiating beam. For samples that contain water, SEM observations typically require preprocessing steps such as drying or freezing the sample to prevent it from suffering shape distortions under high-vacuum conditions. However, there are a variety of situations in which it may not be desirable to observe a sample in a desiccated or frozen state, but rather to observe the dispersion and behavior of materials in liquids, as well as the morphology of *living* cells and microorganisms with the high resolution provided by SEM. This motivated the development of a liquid-sample observation technique using capsule-shaped sample holders to allow SEM observations while preserving sample solutions; these holders were formed from silicon-nitride membranes through which the electron beam could pass, and liquid samples injected into the interior of these holders could be maintained under atmospheric pressure. Observations using these holders employed high accelerating voltages and high-current electron beams capable of penetrating the membranes to irradiate the sample. The resulting transmitted and backscattered electrons were detected by detectors and used to generate images. However, this approach suffered from a number of drawbacks, including a high risk of sample damage due to electron-beam irradiation and difficulty in achieving high-contrast images for samples containing only light elements.

To remedy these shortcomings, Dr. Toshihiko Ogura of Japan's National Institute of Advanced Industrial Science and Technology (AIST) developed a new observation technique capable of imaging liquid samples without the need for high accelerating voltages or high electron-beam currents. Hitachi High-Tech then partnered with Dr. Ogura to develop a system for observing liquid samples, and this system—known as the *Vitro detector*—has been commercially available since 2023. In this paper, we first describe the principles of this new detector, and then present a series of sample measurements demonstrating its key advantages.

2. The Vitro Detector: Structure and Operation

2-1. Structure of the detector

The Vitro detector comprises two components: the Vitro holder and a connector unit placed on the stage. Figure 1 shows a photograph of the Vitro holder and a schematic diagram of its cross-sectional structure. The liquid sample is sandwiched between two silicon-nitride membranes, the upper of which is coated by a layer of heavy metal; this upper membrane, together with O-ring seals, serves to isolate the sample from the vacuum environment, thus allowing the sample to be maintained under atmospheric pressure without drying. Positioned immediately beneath the lower membrane is a detector electrode connected to a preamp; this preamp, in turn, connects to the connector unit, from which the signal is acquired. To prepare a liquid sample for observation, the upper silicon-nitride membrane is turned upside down and droplets of the liquid sample are dripped on it from above. Then the lower membrane is put in place—sandwiching the liquid between the two membranes—and the assembly is turned right-side-up and mounted in the Vitro holder. This allows samples to be observed under high-vacuum conditions in their liquid state without drying.

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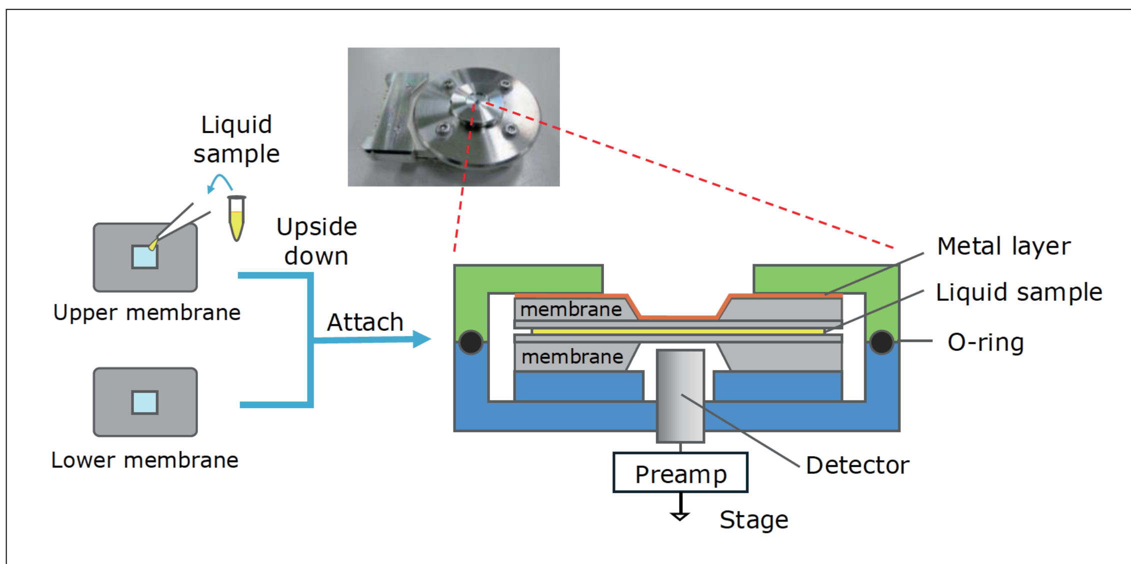

Fig. 1 Vitro holder (inset) and schematic diagram of its cross-sectional structure.

2-2. Principles of operation and key features

The signal-detection mechanism for the Vitro detector is illustrated schematically in Figure 2. Because the liquid sample is in contact with the lower surface of the heavy-metal-coated upper silicon-nitride membrane, applying a bias voltage to the heavy-metal layer creates an electric-field intensity distribution within the upper membrane that reflects the state of the sample. When the assembly is irradiated from above by a low-energy electron beam, the incoming electrons lose most of their energy due to scattering within the heavy-metal layer. Scattered electrons arriving at the silicon-nitride membrane then give rise to local variations in the electric potential, which are transmitted downward through the liquid sample and detected in the form of an electrical signal by the electrode beneath the lower membrane. The intensity of this signal depends on the electric-field intensity within the upper silicon-nitride membrane, and thus reflects the state of the liquid sample between the membranes.

Because this signal-detection mechanism does not require a high accelerating voltage or a high electron-beam current, samples observed with the Vitro detector are less likely to suffer damage due to electron-beam radiation. Moreover, because this approach does not require direct interaction with the sample, it is capable of producing high-contrast images, without staining or fixation, even for samples containing only light elements.

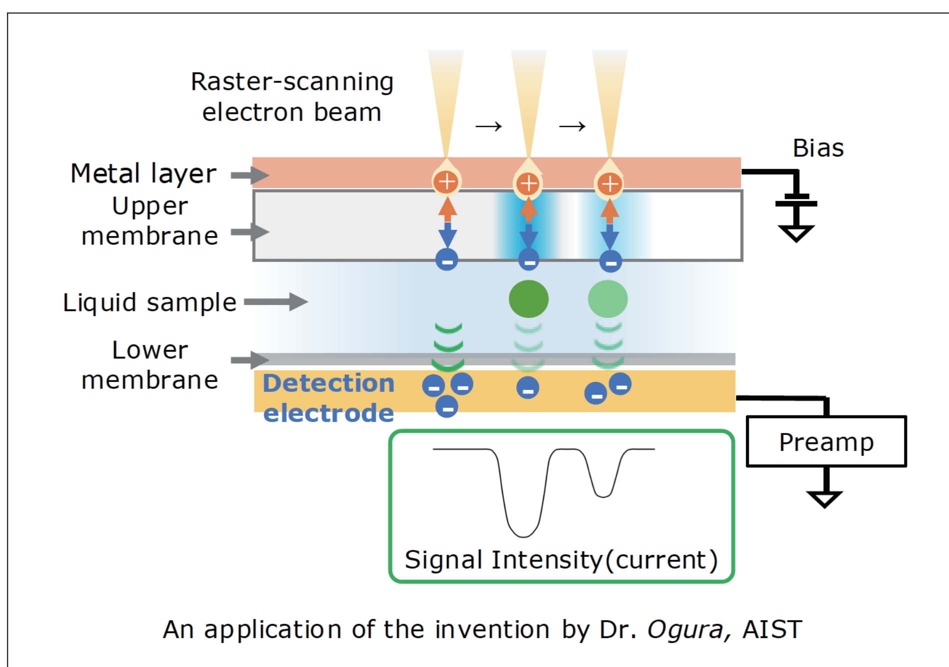

Fig. 2 Operating principle for Vitro detector.

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3. Practical Applications: Examples of Challenging SEM Observations Made Possible by Vitro Detector

We now present examples of actual measurements in which the use of the Vitro detector enables high-quality SEM imaging of liquid samples.

3-1. Food products

For milk and other dairy products, it has traditionally been difficult to obtain high-quality SEM images of samples in their natural liquid state. When attempting to capture images using backscattered electrons or other conventional signals, the damage caused by electron-beam irradiation has the effect of distorting the structure of milk fats as the scan proceeds. To illustrate how the Vitro detector solves this problem, Figure 3 shows SEM images of dairy products—specifically, milk (A,C) and powdered infant formula (B), observed using the Vitro detector (A,B) and the conventional secondary-electron detector (C) at magnifications of 10,000× (A-1,B-1,C-1) or 20,000× (A-2,B-2,C-2) [1]. Samples for observation were prepared simply by injecting the dairy products in their natural liquid state into the Vitro holder. In images A-1, A-2, B-1 and B-2, white spherical structures with sizes of 150 nm to 3 μm are dispersed throughout the aqueous medium (w), and we interpret these to be milk fats (f). Images A-1 and A-2 also show an abundance of black particles with structures similar to those previously reported[2]; we interpret these to be casein micelles (c). A small number of black particles (p) are also visible in image B-2 for the powdered-milk sample. Because powdered milk is produced from milk containing casein protein, it is possible that these particles are also casein micelles. The smaller number of such particles in powdered milk may be evidence that the casein-protein content of powdered infant formula is intentionally reduced to levels similar to that of breast milk because it is difficult for infants to digest and absorb casein protein[3,4]. The clarity of the Vitro-detector images (A) is to be contrasted with the murkiness of the backscattered-electron images (C). Although the latter were captured from the same sample and at the same time as the Vitro-detector images, their quality is not sufficient to distinguish fatty components, casein micelles, or other structures.

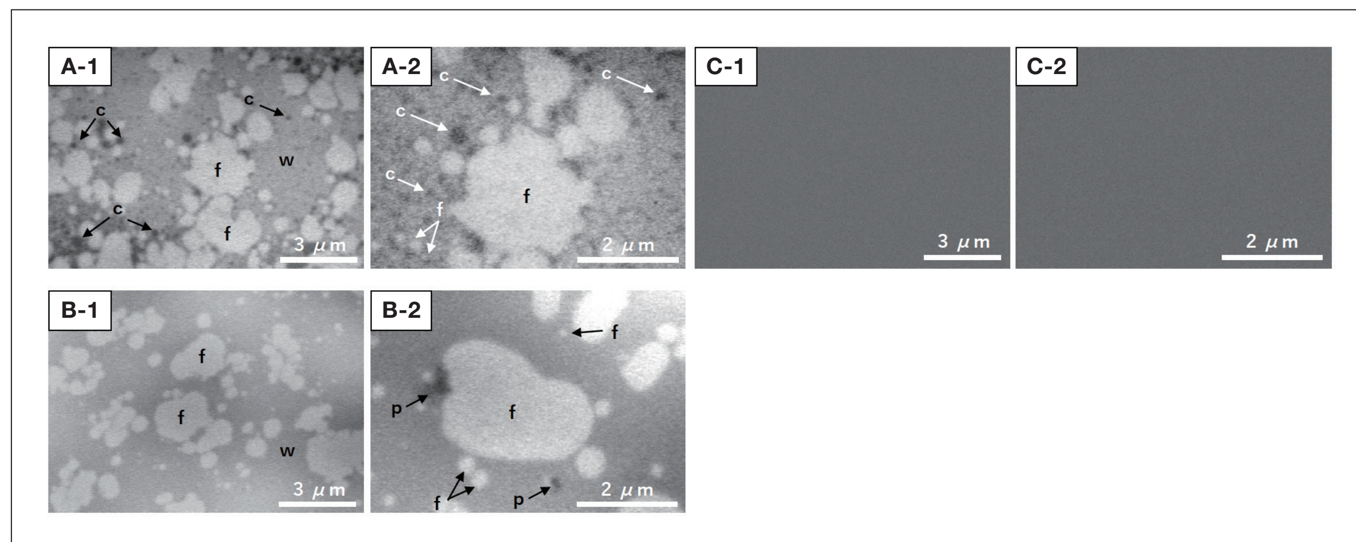

Fig. 3 SEM images of liquid dairy-product samples. A-1, A-2: Vitro-detector images of milk sample. B-1, B-2: Vitro-detector images of powdered infant-formula sample. C-1, C-2: Secondary-electron images of milk sample, captured simultaneously with the Vitro-detector images in A-1 and A-2. Instrument: SU5000. Accelerating voltage: 5 kV.
Magnification: 10,000× (A-1, B-1, C-1); 20,000× (A-2, B-2, C-2).
f: Milk fats. w: Water. c: Casein micelles. p: Particles.

3-2. Liposomes

Our next example involves SEM observation of liposomes. Liposomes are vesicles formed from phospholipids whose ability to encapsulate pharmaceutical substances or other active ingredients makes them promising candidates for applications to drug-delivery systems. To date, electron-microscopy observations of liposomes have employed techniques such as cryotransfer or negative staining[5-7]. Figure 4 shows an SEM image of liposomes captured using the Vitro detector. Note that this approach can successfully image liposomes of many sizes—from tens of nanometers

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to 500 nm—dispersed in a buffer liquid. Other types of biological samples have been studied by Dr. Ogura, who successfully observed liquids containing photosynthetic bacteria, protein antibodies, cultured cells, and other specimens[8,9]. As noted above, a key advantage of the Vitro detector is its ability to achieve high-contrast observations—even for biological samples containing only light elements—without staining or fixation.

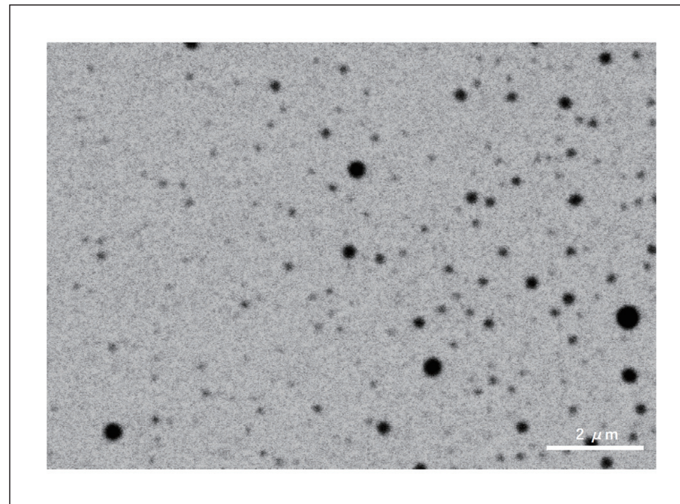

Fig. 4 SEM image of liposomes in liquid. Instrument: SU5000. Accelerating voltage: 3.5 kV. Magnification: 10,000×

3-3. Cosmetic lotion

Our final case study is an observation of a cosmetic-lotion sample. Cosmetic lotions are typical examples of emulsions; like the dairy products discussed above, emulsions contain abundant oil components that make them highly susceptible to structural distortion under electron-beam irradiation. Figure 5 shows a Vitro-detector SEM image and energy-dispersive X-ray spectroscopy (EDS) mapping results for a sunscreen sample prepared simply by pouring the liquid sunscreen into the Vitro holder[10]. In the Vitro-detector image in Figure 5A, oil components (o), water (w), small particles (sp), and large particles (lp) can be observed. Figure 5B shows the corresponding EDS mapping results for the same field of view, obtained using an EDS detector (QUANTAX FlatQUAD, Bruker) positioned directly above the sample. The EDS analysis detects carbon (C) in oil components, oxygen (O) in water regions, zinc (Zn) in small particles, and titanium (Ti) in large particles. This example demonstrates that the combination of the Vitro detector and an EDS detector can yield high-quality SEM/EDS results for samples in their natural liquid state with minimal sample damage even for emulsions or other substances susceptible to damage under electron-beam irradiation.

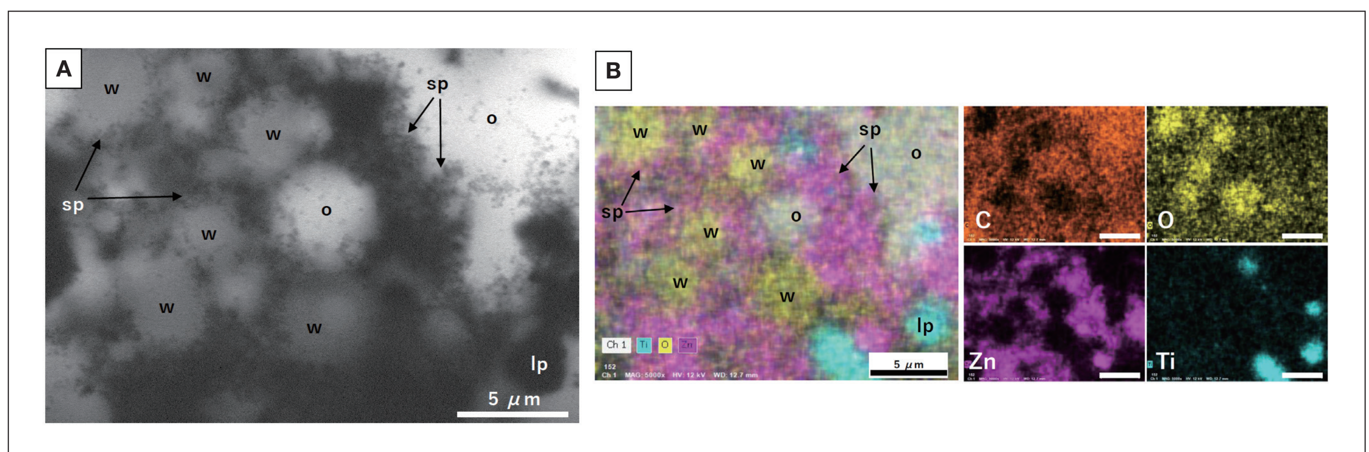

Fig. 5 SEM/EDS observation and analysis of sunscreen lotion. A) Vitro-detector image. B) EDS mapping image. SEM: SU5000. EDS: QUANTAX FlatQUAD (Bruker).
Accelerating voltage: 12 kV. Magnification: 5,000×.
o: Oil components. w: Water regions. sp: Small particles. lp: Large particles.

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4. Conclusions

The Vitro detector is a new type of SEM detector now available for use with Hitachi SEM systems. This article described the operating principles for this detector and presented observation case studies demonstrating its unique capabilities. Key features of the Vitro detector include the following:

• It enables SEM observation of liquid samples.
• It reduces the damage to liquid samples caused by electron-beam irradiation.
• It is capable of producing high-contrast images without staining, even for samples composed entirely of light elements.

Going forward, we expect the Vitro detector to yield valuable new insights that will drive advances in the observation and analysis of liquid samples in the fields of biology, food science, and materials science.

Acknowledgements

In writing this manuscript, and in obtaining the experimental data presented above, we received invaluable suggestions and guidance from Dr. Toshihiko Ogura of the AIST Health and Medical Research Institute, to whom we extend our deepest gratitude.

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Introducing the TM4000PlusIII Tabletop Microscope

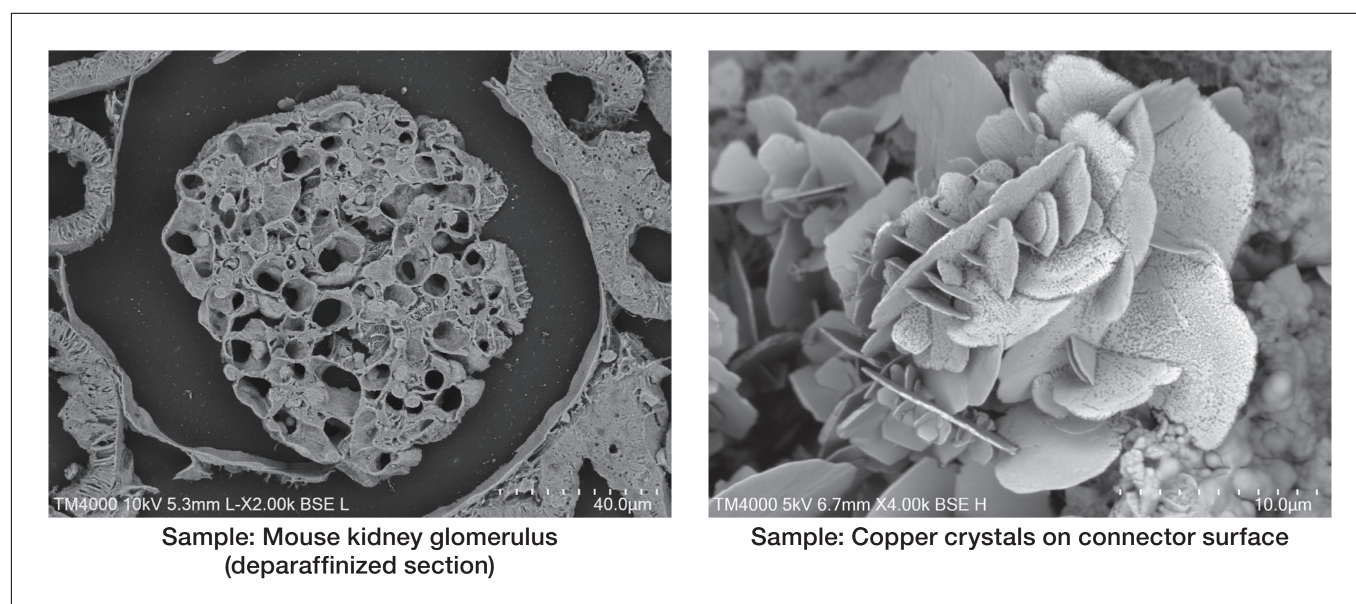
Takeshi Goto

1. Introduction

Scanning electron microscope (SEM), instrument for observing the fine-grained structure of sample surfaces, has become widely-used tools spanning a broad range of industrial sectors, from materials science to biotechnology. Given the growing diversity of this user base, the trend in SEM technology has been toward increasingly complex systems offering wide-ranging capabilities tailored to specific fields and objectives. It was against this backdrop that Hitachi chose to develop a new type of SEM system, occupying a smaller physical footprint and presenting a simplified control interface, that would be easy to use even for first-time SEM users. This was the origin of the Miniscope series of tabletop microscopes, which went on to find applications not only in R&D settings, but also for quality control at manufacturing sites and as educational tools in school science classrooms.

Instruments in the Miniscope series—whose development was guided by the motto *cutting-edge microscopes, made friendlier and easier to use*—were electron microscopes designed to be as easy to use as optical microscopes. This vision, which represented a reversal of conventional paradigms, was received enthusiastically. In the years since the release of the first-generation TM-1000 instrument in 2005, more than 5,000 Miniscope systems have been shipped to customers in Japan and overseas.

Most recently, 2024 witnessed the release of the 7th-generation instrument in this series: the Miniscope TM4000PlusIII. In this article, we survey the key features of the TM4000PlusIII and present a number of applications illustrating its capabilities.



Sample: Mouse kidney glomerulus (deparaffinized section)

Sample: Copper crystals on connector surface

Fig. 1 Examples of images obtained using the TM4000PlusIII.

2. Overview of the TM4000PlusIII

In recent years, Miniscope instruments have been used to develop environmentally-conscious materials and processes for producing them, as well as to analyze air pollutants and toxins in workplace environments—thus helping preserve the global environment and protect human health. At manufacturing sites, the ongoing trend toward the miniaturization of materials, together with increasingly stringent quality-control standards and growing lists of new regulations, have increased the number of sites at which products must be observed via SEM. One consequence has been to expand the pool of SEM users, which is no longer limited exclusively to expert practitioners; instead, the duties

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of maintaining and operating tabletop microscopes for observational tasks are increasingly shouldered by individuals with a broad range of backgrounds.

In view of this development, modern SEM instruments must be capable of producing high-quality results that do not depend on the expertise of the instrument operator—and that do not vary from one operator to the next. This requires streamlining observation workflows and simplifying instrument controls, and the TM4000PlusIII was developed with these goals in mind.

The TM4000PlusIII also carries forth a tradition dating back all the way to the original Miniscope instruments: it is specifically intended for use as a tool to support science education, including efforts to dissuade students from abandoning the study of science. Thus the TM4000PlusIII is not simply an instrument for microscopic observations, but also incorporates new features allowing the system to be used as an advanced educational tool.

Fig. 2 The TM4000PlusIII main unit.

3. Key Features of the TM4000PlusIII

3-1. Streamlined observational workflow with labor-saving features

The TM4000PlusIII features new capabilities to assist in automating observation workflows. These tools allow sequences of observation procedures such as translating the stage, adjusting the magnification, and capturing images, to be saved in the form of *recipes*. Once a procedure has been recorded, it may be executed automatically with just a few clicks. In addition to streamlining observations, this has the advantage of making the instrument more approachable to novice users by making the same observation techniques accessible to all users, regardless of their familiarity with sophisticated analytical systems. This is particularly valuable for users whose duties include lengthy lists of tasks unrelated to making observations, as well as users intimidated by the complexity of configuring observation conditions.

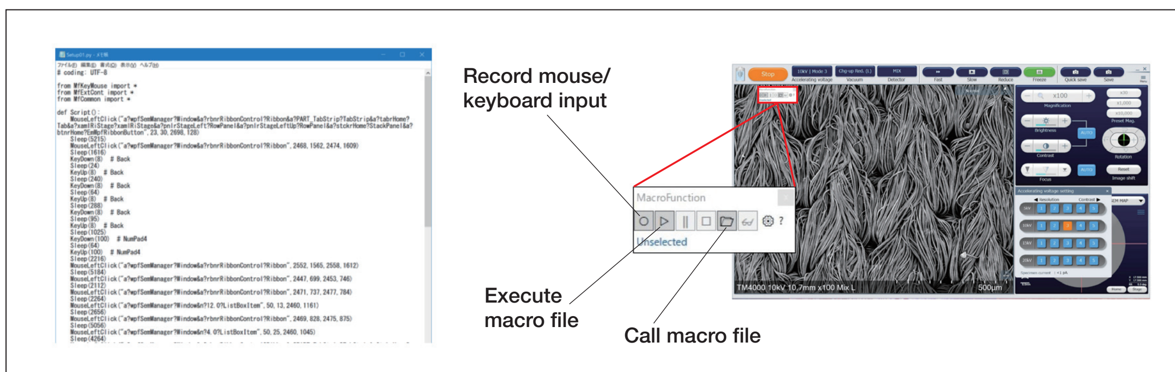

Fig. 3 The TM4000PlusIII's capabilities to assist users in automating observation workflows.

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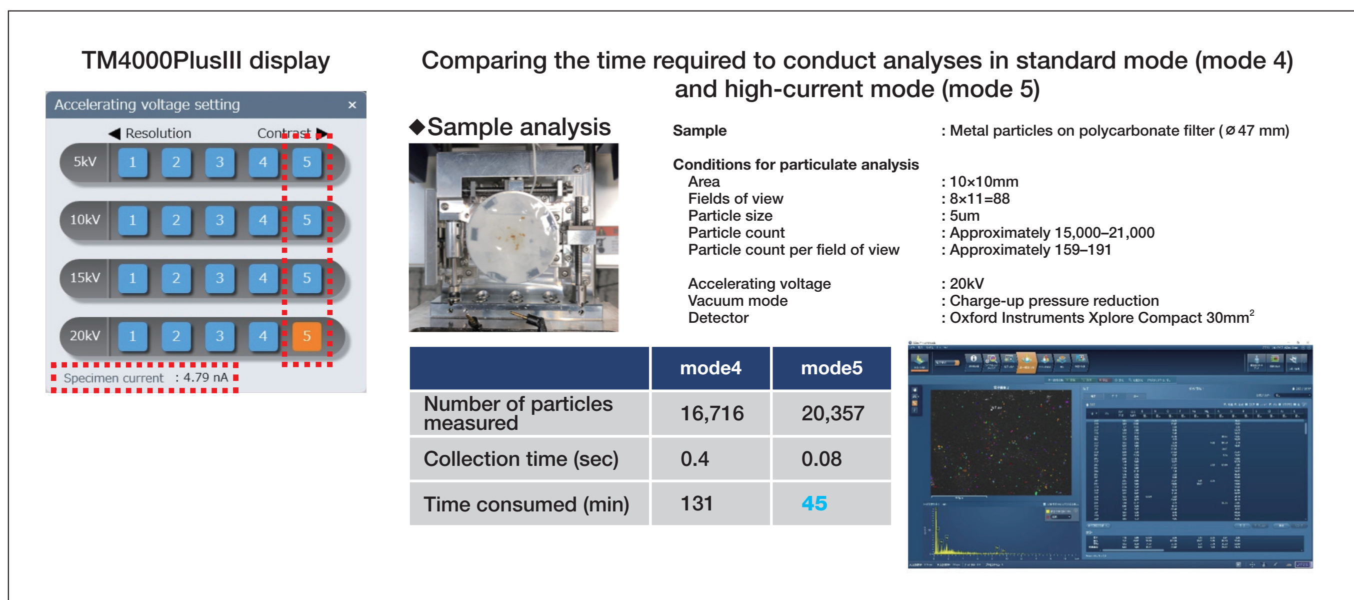
The TM4000PlusIII also boasts two new capabilities to facilitate stable, high-speed measurements covering wide areas: (1) a high-current mode, and (2) a display showing the magnitude of the beam current irradiating the sample. These features shorten measurement times and allow checks on the stability of the beam current. As a result, procedures requiring time-consuming observations at a large number of measurement points—such as quantifying the cleanliness of industrial products or performing automated particulate analysis of replacement filter components—can now be carried out more rapidly with good reliability and less effort on the part of human operators.

The figure below shows results obtained by using the instrument to analyze microscopic impurities trapped in a filter. In standard-current mode (mode 4), the instrument requires more than 2 hours to analyze about 16,000 particles. By contrast, in the new high-current mode (mode 5), the instrument can analyze about 20,000 particles in just 45 minutes.

This analytical technique is likely to yield significant speedups for cleanliness-control processes based on ISO16232/VDA19, which are implemented at production plants for automotive components.

Meanwhile, when conducting repeated measurements during inspection processes, the new ability to check the beam current with just a glance at the instrument screen will allow instrument operators to monitor the beam current in real time, and note any major fluctuations that may occur.



	mode4	mode5
Number of particles measured	16,716	20,357
Collection time (sec)	0.4	0.08
Time consumed (min)	131	45

Fig. 4 Verifying the effects of the new high-current mode in automated analysis of impurity particles.

3-2. Features to support dependable system operation

The TM4000PlusIII offers a new support feature—the *filament indicator*—designed to maximize uptime and instill confidence that the system will be available for use at any time.

A key consumable in any electron microscope is the *filament* used as an electron source. The TM4000PlusIII new filament indicator is an on-screen display allowing users to see at a glance when the filament will need to be replaced. To understand the utility of this feature, note that the filaments used as electron sources in conventional SEMs are constructed in such a way as to exhibit an unfortunate tendency to fail suddenly. Moreover, recognizing the signs that a filament is about to fail is a skill available only to seasoned practitioners after years of continual instrument use. Consequently, attempts to execute sequential observations spanning long periods of time have often been interrupted by sudden filament failures demanding urgent filament replacement. The filament indicator eliminates such catastrophes by allowing users to check—*before* initiating an observation—whether the filament is near the end of its useful lifetime and should be replaced to avoid interruption.

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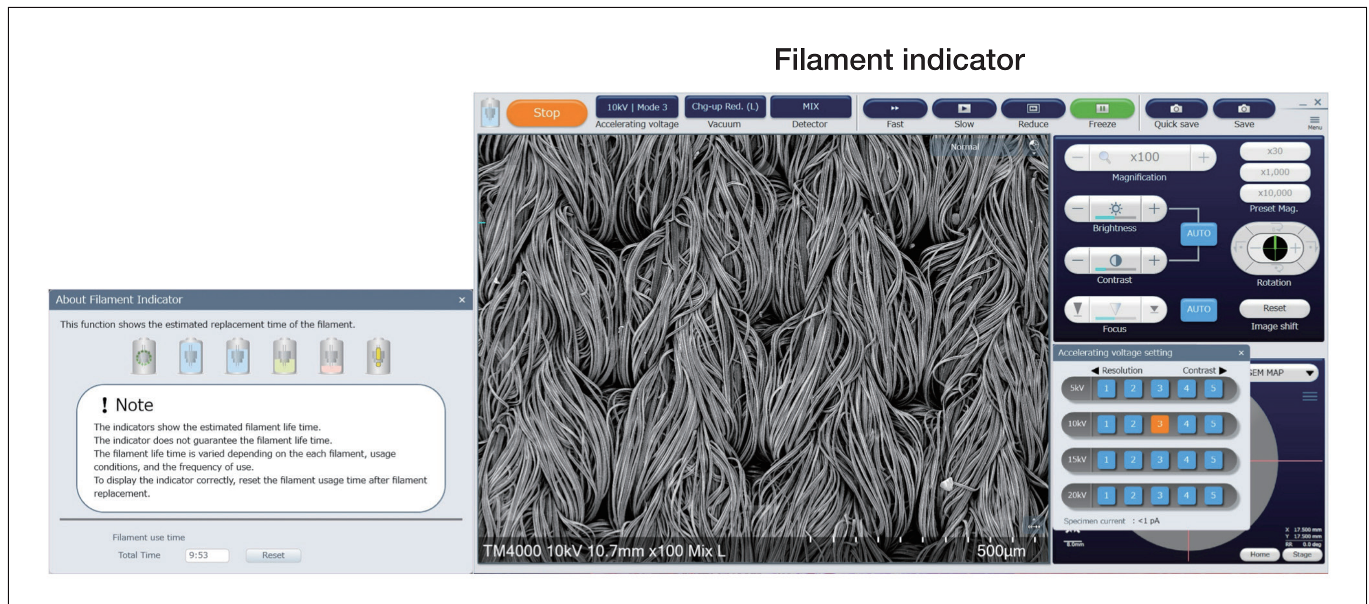

Fig. 5 New feature of the TM4000PlusIII: filament indicator

3-3. The TM4000PlusIII as a new tool for teaching computer programming

Like other instruments in the Miniscope series, the TM4000PlusIII supports *low-vacuum* observations. Such observations simplify the task of preprocessing samples and are relatively easy to perform, making them appropriate for applications in educational settings.

In Japan, training in digital skills is considered an important goal for educational institutions—for example, "Information I" course is a mandatory component of the high-school curriculum. The Python scripts used to assist in automating observation workflows in the TM4000PlusIII offer a valuable opportunity to promote this type of education: by writing Python programs to control the TM4000PlusIII, students acquire first-hand experience while learning key programming concepts such as sequential execution, looping, and conditional branching.

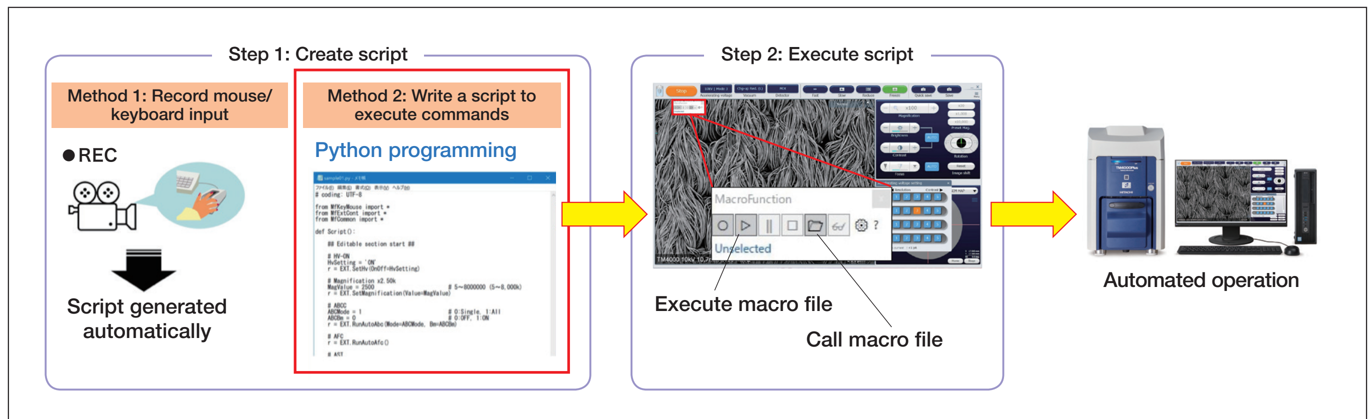

Fig. 6 Using macros to define operational procedures.

4. Conclusions

The new features of the TM4000PlusIII for assisting the automation of observation workflows will reduce the effort required to operate the system—and level the playing field to allow operation by users with varying levels of technical expertise. Furthermore, the addition of programming capabilities to automate operational commands will allow the TM4000PlusIII to serve as a powerful educational tool for training future generations of skilled professionals. We expect this new system to be widely adopted as a product capable of meeting a diverse spectrum of customer needs.

Going forward, Hitachi High-Tech will continue not only to advance the capabilities of observation and analysis systems, but also to meet the needs of the increasingly diverse community of electron-microscope users.

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Application of X-ray Fluorescence Analysis to the Characterization of Recycled Materials

Atsuko Yamada

1. Introduction

Modern mass-production, mass-consumption societies discard enormous quantities of unneeded waste products on a daily basis. Failure to process these waste products appropriately could pose grave environmental problems; we must therefore pursue the goals of *reducing*, *reusing*, and *recycling* waste. Figure 1 illustrates common processes for handling various types of waste. As this figure suggests, techniques for making effective use of waste products—and the analytical tools required by those techniques—differ depending on the type of waste and the relevant processes. In this article we focus specifically on *ash* and *slag*.

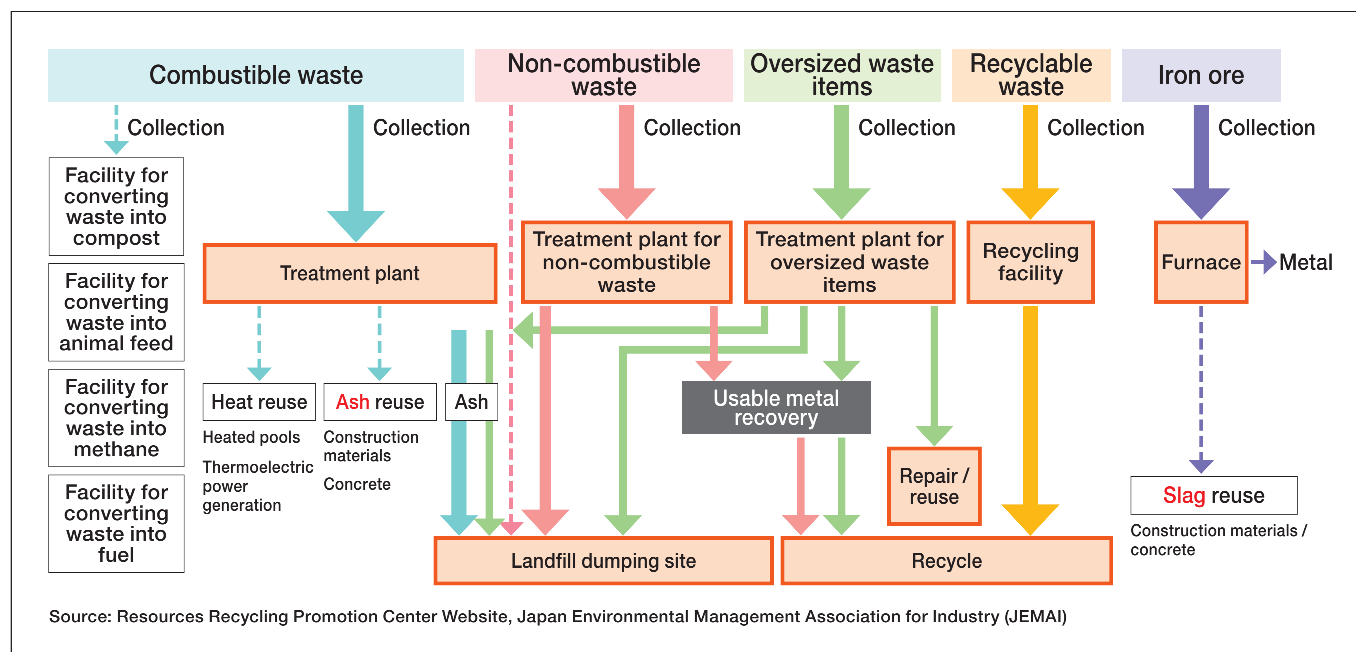

Fig. 1 Processes for handling waste products.

Ash and slag may be reused to produce concrete or other construction materials, but this requires analysis and maintenance procedures to verify that the material composition is suitable for this purpose and that toxic-element concentrations are below regulatory thresholds. Methods for performing these analyses are specified by two industrial standards: JISM8815:1976, "Methods for Analysis of Coal Ash and Coke Ash," and JISK0058-2:2005, "Test Methods for Chemicals in Slags, Part 2: Test Method for Acid Extractable Contents of Chemicals." However, the procedures described in these standards involve complicated operations and pre-processing steps, making them unsuitable for rapidly analyzing large numbers of test samples. To overcome this problem, screening methods based on energy-dispersive X-ray fluorescence analysis—which requires no complicated preprocessing, allows analysis of solid, liquid, and powder samples, and can perform non-destructive analysis and simultaneous multiple element measurement—are frequently used[1).

2. Energy-Dispersive X-Ray Fluorescence Analyzer EA1400

In this study, we analyzed ash and slag using the EA1400 energy-dispersive X-ray fluorescence analyzer from Hitachi High-Tech Science. The EA1400 is equipped with an X-ray-emitting vacuum tube and a semiconductor detector positioned beneath the sample stub; after mounting a sample, a measurement lasting just a few minutes

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suffices to allow qualitative and quantitative analysis of the elements (from $_{11}Na$ to $_{92}U$) contained in the sample. Boasting a new high-resolution, high-count-rate silicon drift detector (SDD) and vacuum system that enable higher-sensitivity, higher-throughput measurements than existing energy-dispersive X-ray fluorescence analyzers, the EA1400 is widely used for quality control and screening of regulated elements, and has also been employed for a broad range of other purposes, including environmental studies and analysis of cultural artifacts.

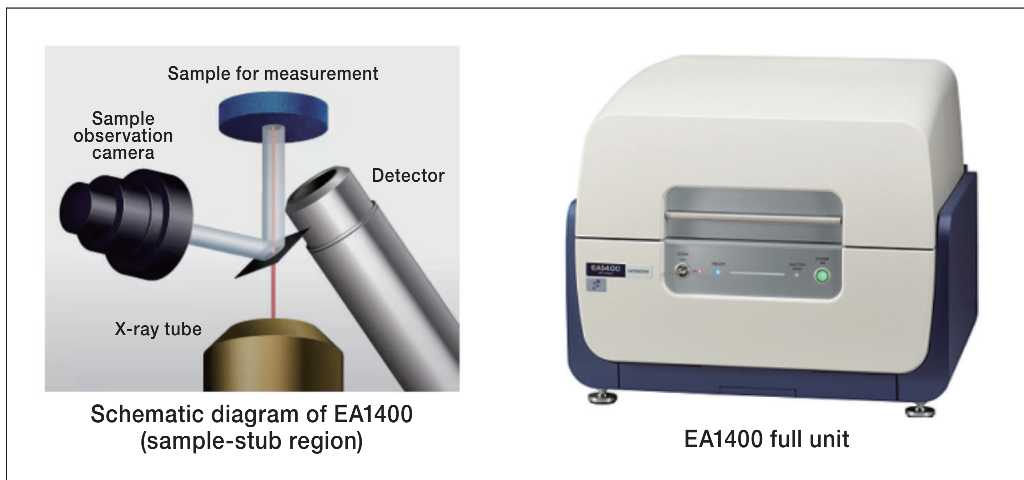

Fig. 2 Energy-dispersive X-ray fluorescence analyzer EA1400.

3. Samples and Measurement Conditions

The ash sample we used was a reference sample of coal ash (certified reference material JSAC 0522) from the Japan Society of Analytical Chemistry. The slag sample was a water-granulated slag that we obtained independently and crushed to ensure particle diameters below 0.25 mm. We prepared two sample cups— of inner diameter 24 mm and height 22 mm with polyethylene films affixed to the lower surfaces—to which we added approximately 10g of each sample, and then covered with paper lids. We measured each sample using the EA1400; to increase sensitivity to fluorescence X-rays from elements with low atomic numbers (up to $_{20}Ca$), we used a rotary pump to evacuate the specimen chamber to a vacuum state and set the measurement time to 400 seconds.

Table 1 Experimental conditions used to measure samples of coal ash (certified reference material JSAC 0522) and water-granulated slag.

Instrument	EA1400 (Rhodium X-ray tube)			
Method for computing elemental concentrations	Method of bulk fundamental parameters			
Measurement time (s)	100	100	100	100
Collimator diameter (mm)	Φ 5.0	Φ 5.0	Φ 5.0	Φ 5.0
Excitation voltage (kV)	15	15	50	50
Tube current (μA)	Set automatically	Set automatically	Set automatically	Set automatically
Primary filter	None	For Cr	For Pb	For Cd
Polyethylene film affixed to sample cup	Yes	Yes	Yes	Yes
Environment	Vacuum	Vacuum	Vacuum	Vacuum

4. Results and Discussion

The measurement results obtained using the EA1400 are listed in Table 2. For the coal-ash sample we compared EA1400 measurement results to standard tabulated values for this certified reference material (JSAC 0522). For the slag sample we compare EA1400 measurement results to those obtained using an inductively-coupled-plasma optical-emission spectrometer (ICP-OES).

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Table 2 Results of EA1400 measurements compared to standard tabulated values (for coal-ash sample) or to ICP-OES measurements (for slag sample).

Comparison of EA1400 measurements and standard tabulated values for coal-ash sample (certified reference material JSAC 0522)

	Primary constituent mass fraction (%)							Trace components (mg/kg)	
	SiO_2	Al_2O_3	TiO_2	CaO	MgO	P_2O_5	K_2O	Cr	Pb
Standard tabulated values	59.4	28.7	1.7	1.3	0.60	0.33	0.29	139	98
EDXRF* (EA1400)	58.4	32.4	1.8	1.4	0.75	0.55	0.33	140	97

*EDXRF: Energy dispersive X-ray analysis

Comparison of EA1400 and ICP-OES measurement results for water-granulated slag sample

	Primary constituent mass fraction (%)								
	CaO	SiO_2	Al_2O_3	MgO	S	TiO_2	FeO	K_2O	MnO
ICP-OES	41.0	32.2	14.5	5.8	1.1	0.63	0.33	0.32	0.24
EDXRF(EA1400)	42.9	33.5	15.6	4.5	1.5	0.62	0.52	0.45	0.26

The EA1400 measurement results closely match standard tabulated values (for the coal-ash sample) or ICP-OES measurements (for the slag sample); we attribute this good agreement to the meticulous care we have taken to ensure satisfaction of the key requirements summarized in Table 3.

Table 3 Key requirements for successful X-ray fluorescence analysis

Requirement	Ideal sample	Non-ideal sample	Strategy used in this study (for ash or slag)
Sample homogeneity	Homogeneous, uniform	Inhomogeneous, non-uniform	Crushed
Sample shape/ thickness	Planar slab (of sufficient thickness to constitute a bulk region)	Granular Thin sheet Elongated · ·	Sample cups of appropriate size and shape used to hold samples (thickness of sample in sample cup: ≥ 5 mm)
Elements contained in sample	Sample comprised solely of measurable elements	Resins, oils, aqueous solution Oxides, nitrides · ·	For theoretical calculations of expected X-ray fluorescence intensities (based on fundamental parameters), we assume that all measured elements present in samples exist in the form of their typical oxides

A first essential prerequisite for successful X-ray fluorescence analysis is to use observation samples of homogeneous composition; it was for this reason that we crushed the water-granulated slag to yield homogeneous slag samples for EA1400 measurements. The sample volume we chose (10 g) was designed to ensure that each sample would have thickness sufficient to constitute a bulk region. This is considered to be the case if varying the sample thickness does not affect its fluorescence X-ray intensity. The minimal thickness required depends on the primary constituents of the sample and on the fluorescence X-ray energies for the elements measured; for ash and slag samples, it is around 5mm.

In this study, we quantified elemental concentrations using the *method of fundamental parameters* (FP): If the composition of a sample (all elements contained in the sample and their relative abundances) is known, the expected intensities of fluorescence X-ray emissions may be computed theoretically—based on the principles of fluorescence X-ray emission—in terms of experimental parameters and physical constants (or “fundamental parameters.”) The FP method determines the composition of a sample by comparing these calculated theoretical intensities to experimentally measured emission intensities for the sample.

Although there are formulas for converting measured intensities directly to element abundances, these formulas are complicated and difficult to use; instead we use a method of successive approximations, which proceeds as follows. We begin by determining an initial estimate—a starting guess—for the sample composition, then compute the fluorescence X-ray intensities for this composition and compare them to the measured intensities. If the computed and measured intensities greatly disagree, we adjust our composition estimate appropriately, then re-compute the expected

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intensities and compare to measured intensities. Continuing in this way, we repeatedly adjust our composition estimate until the discrepancy between the computed and measured intensities is sufficiently small; the composition estimate for which computed and measured intensities agree is then returned as the result of the analysis[2).

The primary elemental constituents of both ash and slag are known to exist in the form of oxides; thus, in this study we assume that the primary elements for which we measure fluorescence X-rays exist within samples in the form of their typical oxides. From Table 3 it can be seen that, by taking pains to ensure that all key requirements are satisfied, the EA1400-based analysis succeeds in determining accurate concentrations not only for the *principal* components of coal ash and water-granulated slag, but also for the *trace* components (Cr,Pb) present in coal ash. These results demonstrate that X-ray fluorescence analysis, despite being a highly sensitive tool, is nonetheless capable of yielding analytical results that closely match those obtained via the analytical procedures specified by industrial standards—and will likely prove a powerful technique for analyzing recycled materials.

5. Conclusions

Energy-dispersive X-ray fluorescence analysis requires no complicated preprocessing, allows analysis of solid, liquid, and powder samples, and non-destructive analysis and simultaneous multiple element measurement—and is thus a powerful analytical tool for screening recycled materials, an application demanding rapid analysis of large numbers of test samples. In this study we demonstrated that X-ray fluorescence analysis—with careful attention paid to ensuring the satisfaction of certain key requirements—can yield results similar to those obtained via the analytical procedures specified by industrial standards. We expect that X-ray fluorescence analysis, a technique already used in a broad variety of fields, will also become a widely-used tool for analyzing recycled materials.

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A Fateful Encounter with SEM: Everything Revolves Around *Visualizing Phenomena*

An Expert in Measurement and Analysis—with a Multi-decade Track Record Supporting TOTO Product Development

The TOTO Corporation's (hereinafter referred to as TOTO) Comprehensive Research Institute has been conducting extensive research to make water-related spaces in daily life cleaner and more comfortable. Additionally, they have achieved significant results in a wide range of fields, such as providing development support for applying ceramic technology to semiconductor production equipment. On this occasion, we visited the Analytical Technology Center of the institute and spoke with Senior Researcher Toshihiro Aoshima, who has been involved in analysis work for many years. We discussed the achievements of TOTO's analysis work and the introduction of ExTOPE, which contributes to data sharing and operational efficiency.

Senior Researcher
Analytical Technology Center
Comprehensive Research Institute
TOTO Ltd.

Toshihiro Aoshima

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INTERVIEW

Transforming household water-related issues into solutions

The name TOTO is likely something everyone has seen at least once in their daily life. Playing a crucial role in the technological development of indispensable products for everyday living is the Comprehensive Research Institute located in Chigasaki City, Kanagawa Prefecture. The mission of this institute, as a research center for a leading company in water-related spaces, is to explore new proposals and technologies that transform everyday issues in toilets, bathrooms, washbasins, and kitchens into positive experiences.

Since its founding in 1917, TOTO has conducted fundamental research on materials and products within its respective business divisions. Later, the company established a research institute, which evolved into the Comprehensive Research Institute in 2002. Today, the institute conducts comprehensive product research for all of TOTO, expanding its scope to include fields such as ergonomics, sensory engineering, lifestyle, and living patterns, creating core technologies for products. One key aspect of core technology development is analytical technology. Currently, the development of materials at the nano level is a critical issue across all industrial fields. For TOTO, analytical technologies that clarify invisible elements like water quality, materials, odors, and microorganisms are directly linked to the development and offering of attractive products.

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The start of a technical career with the encounter of X-560

Toshihiro Aoshima joined the company in 1978 and began his career in the analysis group of the laboratory, which was the precursor to the research institute launched in 1979. Since then, Aoshima has consistently tackled important issues in TOTO's research and development and within its various business divisions. He reflects on his experience: "The history of the research institute began in a small building at the western edge of this Chigasaki factory. It was in the basement there that I encountered the Hitachi X-560, which I now think of as a fateful meeting. The X-560 was a localized analysis device equipped with SEM and WDX (wavelength-dispersive X-ray spectrometer), combining observation and elemental analysis. Unlike the automated devices of today, it was the ultimate manual device that helped hone observation techniques and the sensitivity for localized analysis.

In 1980, the company experienced a significant leap forward, marked by the launch of the Washlet, which became synonymous with bidet toilet seats. Furthermore, in 1984, the Ceramic Division was established, and since then, the institute has deepened its understanding of phenomena through various analytical technologies, working closely with the division.

The evolution of analytical devices and the enhancement of research and development capabilities

Over time, the analysis group expanded its staff and continuously introduced cutting-edge analytical devices. "Introducing new equipment provided opportunities to interact with development engineers from analytical device manufacturers and researchers from academia, fostering growth as an analytical technologist. A major turning point was the introduction of the Hitachi S-800," Aoshima recalls. The S-800 was a high-resolution scanning electron microscope (SEM) that achieved the world's best resolution of 2 nm at an accelerating voltage of 30 kV at the time. Its improved operability significantly enhanced the user experience, leading to the rapid spread of FE-SEM technology.

"The first image displayed was of nm-sized tungsten particles deposited on an alumina substrate. As the CRT's raster (a single scan line moving from top to bottom across the screen) slowly moved in the darkness, the beautiful particles shone brightly and vividly. I vividly remember the excitement of seeing such a striking image for the first time, as if it happened yesterday."

Acquiring the S-800 substantially improved TOTO's research and development capabilities, according to Aoshima.

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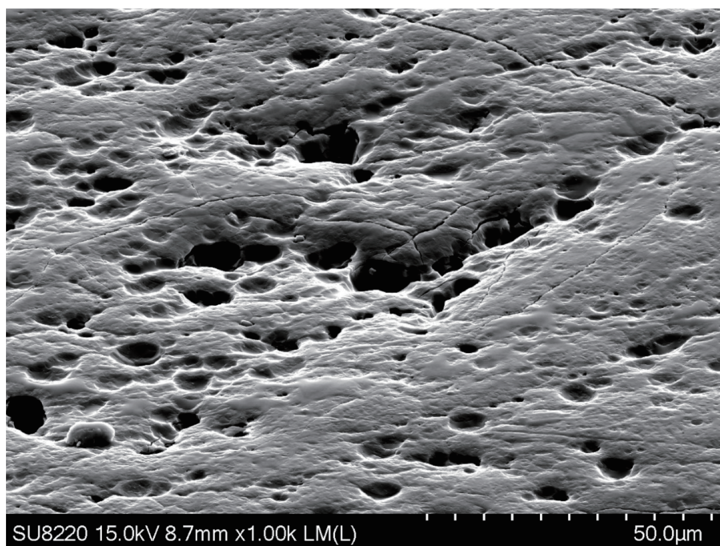
Contributing to cleaner and more comfortable water-related spaces

The work of the Analytical Technology Center contributes significantly to the development of various TOTO products. One example is "CeFiONtect," which remains an important selling point for TOTO toilets. Faced with the challenge of significantly enhancing the toilet's stain resistance, Aoshima, as a member of the development team, observed the surface of aged sanitary ceramics. The observations revealed that dirt accumulates in the zircon roughness exposed by the melting of the glaze. The development team addressed this issue by creating a glass layer without zircon. The resulting CeFiONtect glass layer possesses nano-level smoothness, preventing dirt adhesion and making it easy to clean, with durability confirmed through microscope images for over 10 years of use.

For "Cleanse Water," which is produced by electrolyzing chloride ions in tap water, capturing the process of bacterial death and contributing to the



SEM image of surface of anodized aluminum observed at 60° tilt angle.

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development of durable electrode surface materials were key achievements. In developing water-saving toilets that flush waste with minimal water, X-ray CT was used to create a 3D model of the entire toilet, allowing fluid simulation to visualize water flow, ultimately shortening the development period.

Through these series of clean technology developments, TOTO's sanitary ceramics have achieved water savings to less than one-third of what was used 30 years ago. This not only reduces the use of detergent and water for users but also addresses societal issues by effectively utilizing precious water resources.

Expanding research in ceramics and activities beyond the company

The Analytical Technology Center engages in analytical technology across various phases such as manufacturing, quality assurance, and research, dealing with a wide range of materials. Building on these achievements, Aoshima's activities have broadened to include presentations at academic conferences and collaborative research with academia.

"In joint research with Professor Keisuke Ohta from Kurume University School of Medicine, we worked on elucidating anti-algal mechanisms, clarifying the microstructures of algae, mold, bacteria, and biofilms."

This research expanded into "Hydrotect," leveraging photocatalytic action, and is used in materials for environmental purification, such as building materials and exterior walls.

Additionally, at Kyushu University’s Graduate School, Aoshima has been giving lectures for 18 consecutive years on research and development utilizing analytical technology in the Ceramic Engineering Course, hosted by the Manufacturing Engineering Education and Research Center. This aims to nurture young talent in regional companies, and the course consistently receives high evaluations from participants.

The remote operation of analytical devices with ExTOPE quickly became a collaborative research example. The project, which allows remote operation of a high-resolution electron microscope located in Chigasaki from divisions in Kitakyushu (headquarters), Nakatsu, and Gifu, began with the understanding of the utility of analytical devices at the Comprehensive Research Institute, Aoshima explains. Initially, there were issues with communication response, but now each TOTO division uses it to solve their challenges and develop new technologies. Operating an electron microscope requires expertise; it’s not simply pressing a button to see what you want. Aoshima notes that when division representatives operate the microscope, a brief advice from him can lead to dramatically improved images. Furthermore, on-site guidance during discussions on image interpretation and problem-solving is crucial. ExTOPE seems poised to play a vital role in passing down skilled techniques and nurturing successors.

One notable achievement includes the electrostatic chuck, a key component in semiconductor manufacturing equipment. In the development process, the conductive mechanism was elucidated, leading to a product with excellent plasma resistance, which has become a flagship product of the Ceramic Division.

"The electrostatic chuck is expected to be a new revenue source within the ceramics business, contributing to productivity improvement and cost reduction in the semiconductor manufacturing field. This achievement was recognized with the Technical Award from the Ceramic Society of Japan in 2009, shared by members from the Ceramic Division and the Research Institute. Above all, continuous research is essential to meet the cutting-edge needs of semiconductor manufacturers and to propose future-oriented solutions. Analytical technology that elucidates micro and nano domains holds the key to this pursuit."

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The role of analysis is to reveal the truth

In the fall of 2004, when TOTO's Specialist system began, Aoshima was appointed as the first TOTO Specialist, becoming Chief Researcher. By 2010, he became Senior Researcher, focusing on advising and guiding "analytical technology," "analytical equipment," and developing "analytical talent" to enhance the analytical capabilities of the business divisions.

Aoshima's extensive involvement with analytical device manufacturers, academic activities, and collaborative research with academia undoubtedly stems from his experience as a TOTO Specialist. He has made numerous contributions both internally and externally and asserts that the role of analysis is "to reveal the truth."

"The truth is intricately linked to visible phenomena and properties. What is expected of us in the analysis department is to unravel and clarify this intricate world. Since we are dealing with a truly sophisticated opponent, this technology is naturally profound. Advancing it requires a wealth of experience, know-how, and above all, intuition."

He also describes a specialist as someone who knows their limits. "You understand how a material should appear and have the capability to reach that point. Obtaining beautiful data that can speak volumes with a single photo attached to a report is very important."

Utilize the convenient environment to continue growing

Reflecting on the past, when the S-800 was first introduced, images were captured on film. Technicians at the time processed them in darkrooms, painstakingly developing and attaching the printed photographs to reports with adhesive. These manual tasks served as an excellent classroom for learning principles and are anecdotes that convey how much more fortunate the digital era's analytical environment is, he explains.

"The evolution of analytical devices is not limited to SEM; new methods are continuously being developed, allowing us to vividly see and measure worlds previously unseen or unmeasurable with high sensitivity. I was a demanding user who often told Hitachi, 'I'd like to see this,' 'It would be great to have this feature,' and some of these wishes led to patents. With each evolution of equipment, our analytical technology improved, for which I am grateful."

Aoshima believes that, although the speed of work, the amount of information, and the way we work have changed significantly from his time, the fundamental spirit of a technician should remain the same across eras.

"I hope the younger generation will skillfully utilize the overwhelmingly convenient aspects compared to the past and make full use of their TOTO business cards to grow as corporate technologists. I wish Hitachi would continue supporting the advancement of TOTO's analytical technology," he said with a smile.

In addition to supporting TOTO's development as an analysis specialist, Aoshima has another role as the head coach of the TOTO women's basketball team. The team is a member of the Japan Business Basketball Federation and competes in the regional corporate league. They achieved a long-desired first

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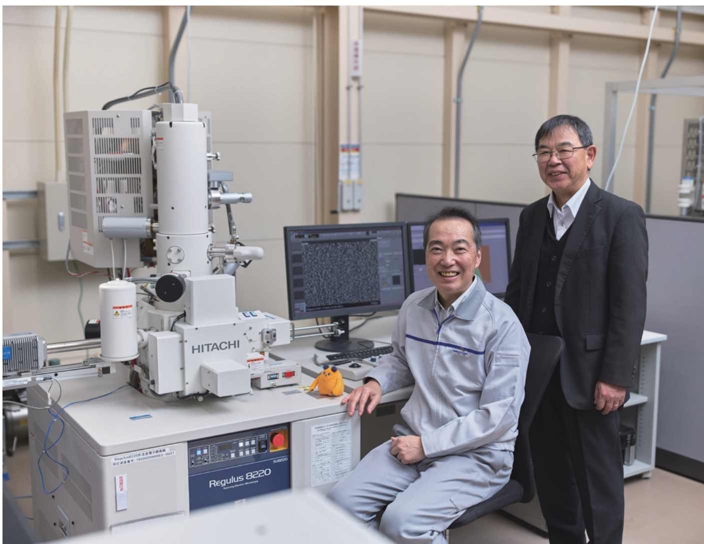
victory in the 2024 Women's East Japan Regional League under the slogan "You be bright this time! ~ Shine now ~." Alongside aiming for victory, Aoshima actively engages with children through mini-basket clinics and experience classes, contributing to the community. This aspect is introduced as part of his character, reflecting his extensive involvement with many people and tackling numerous challenges both inside and outside the company.



Editorial Note

I have had the pleasure of knowing Mr. Aoshima for approximately 30 years through demos, conferences, and seminars. His passion for SEM analysis is remarkable, and I fondly recall the nights spent at a family restaurant in Shonan, deeply engaged in discussions about image interpretation and equipment improvements, as if it were just yesterday. I also remember watching the practice match between the basketball teams of TOTO and Hitachi High-Tech, noticing that his demeanor as a coach was akin to his fervor for SEM. During the recent interview, I learned about his numerous achievements, from the establishment of the Research Institute to various research and development episodes, reaffirming that his recognition as a specialist is well-deserved. My association with Mr. Aoshima, who has adopted many Hitachi SEMs, starting with the X-560, is a valuable asset to me, and I would be honored to continue this relationship in the future. Next time, I hope we can reminisce about the past and discuss the future over drinks at a place with a view of the Shonan sea.

(Ryuichiro Tamochi)

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Biological Control That Contributes to Sustainable Agriculture

Interdisciplinary Research in Ecology and Molecular Biology Aiming for Innovations in Pest Control

As the global population increases at an alarming rate, there are concerns about food shortages in the future. As part of this worrying scenario, farmers across the world are seeing their crops suffer ever greater damage from drought, floods, and pests made worse by climate change. Japan's agricultural sector is also facing problems such as the declining birth rate and aging population, and the quest for increased sustainability has become a pressing issue. With pest control in particular, the problem of resistance to the pesticides that are the main weapon in the fight against pests is becoming increasingly serious, and there is a need to establish new methods of control. Biological control, which makes use of the natural enemies of pests, looks promising as an effective solution. One of the leading researchers in this field is Professor Norihide Hinomoto of Kyoto University Graduate School of Agriculture. We spoke to him about the advantages of biological control, the focus of his current research, and the use of genome sequencing technology in pest control research.

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Pest control using natural enemies

Damage to crops from insect pests has been increasing worldwide in recent years due to the effects of climate change. Professor Norihide Hinomoto of Kyoto University, who specializes in research into pest control technologies, points out that while each individual pest may be diminutive in size, the cumulative effect of pests on agriculture is huge. “It has been calculated that around 40% of the global crop yields that should be possible is lost to pests, which include insects and other animals, diseases, and weeds,” he says. “We are seeing losses on this scale despite measures such as regular spraying with pesticides. To put it differently, if we could prevent this damage, we would be able to increase crop harvests by something like 1.7 times the current yields.”

Spraying pesticides to control insect pests is tough work. In the heat of summer, farmers have to don protective wear such as long sleeves, masks, and goggles, but even after spraying they still suffer huge losses. It seems a thankless task.

“Resistance to pesticides is an enormous problem,” says Hinomoto. “If you keep using the same type of pesticides, the number of pests that can survive gradually increases, and eventually the chemicals don’t work anymore. In Japan, the population is aging as the birthrate decreases, and there is starting to be a shortage of people to take over agriculture from the older generation. For this reason, we need increased labor-saving and efficiency in agriculture. Improving working conditions in agriculture by reducing the burden of pesticide spraying is an important step in encouraging new workers to enter agriculture. Biological control is a promising method for overcoming these problems.”

Chemical pest control prevents pests and pathogens through the use of agro-chemicals with active ingredients that are mostly synthesized by chemical means. Biological control, on the other hand, fights pests by using insects or microorganisms that are the pests’ natural enemies. There are two methods of biological control: one is to purchase natural enemy organisms (biological control agents), which are registered and marketed in the same way as chemical pesticides, and release them on agricultural fields; the other is to attract native natural enemy organisms living in surrounding areas to the agricultural fields. Prof. Hinomoto’s research covers both methods.

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Biological control using natural mechanisms

There is a huge variety of pest species that attack crops, but Prof. Hinomoto's research is focused on the very small types such as spider mites, thrips, whiteflies, and aphids, as well as their natural enemies. "These tiny pests have short lifespans, often around 10 days from egg to adult. This means that over a single year there may be several dozen generations," he explains. "If you continually dose them with a chemical pesticide, the survivors will steadily be selected and in no time at all the pest will have acquired resistance to that particular pesticide. To add to this, environmental standards have become stricter over recent years, and it takes a considerable amount of time and money to develop a new chemical pesticide. As a result, the types of pesticides available for use are becoming increasingly limited."

In 2021, the Ministry of Agriculture, Forestry and Fisheries formulated its "Green Food System Strategy." This strategy aims to bring about the coexistence of increased productivity and sustainability of food, agriculture, forestry, and fisheries, and one of its initiatives is the establishment and spread of comprehensive insect pest management systems that do not rely solely on chemical pesticides, with the aim of a 50% reduction in chemical pesticide use by 2050. The key to achieving this goal is biological control. Prof. Hinomoto is keen to emphasize the significance of this method: "If we can fight pests by making use of the predator-prey relationships and competitive relationships that organisms have built up naturally, it will have a low impact on the ecosystem and will help us to increase the sustainability of agriculture," he says.

However, while pest control using natural mechanisms offers huge advantages, dealing with living organisms is not without its challenges. Prof. Hinomoto explains that it is difficult to estimate and control the timing and quantity of releases: "If you release the natural enemies when the pests are scarce or aren't there, the natural enemies will die of starvation or move elsewhere. If there are too many pests, the natural enemies won't be able to eat them all," he says. "The main focus of my research is how biological control can be used in a stable fashion. Specifically, my research is advancing along the two main lines of clarifying the ecology of the target pests and their natural enemies, and creating high-performance strains of the natural enemy materials for commercialization."

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Participation in a Moonshot Research Project

Among the natural enemies that Prof. Hinomoto is currently researching are the various species of phytoseiid mites that prey on spider mites. Spider mites are parasites of plants, living in groups on the undersides of leaves and sucking the sap from the plant to gain nutrients. If a plant is parasitized by large enough numbers, its growth will be stunted, and it may even wither and die. Spider mites are troublesome pests that are widely distributed, and they parasitize many different types of plant. An important species is the two-spotted spider mite, which in particular has become a massive problem around the world as it readily acquires resistance to pesticides.

In the laboratory, Prof. Hinomoto breeds two-spotted spider mites and their natural enemies, phytoseiid mites, and examines and analyses them in the laboratory environment. He also carries out joint research with research institutions in different regions, analyzing samples and data collected from actual agricultural land.

"I am currently taking part in the Cabinet Office's Moonshot Agriculture, Forestry and Fisheries Research and Development Program, for which I am putting together a research project that aims for the 'Realization of zero pest damage agriculture by fully utilizing advanced physical methods and unused biological functions,'" he explains. "Specifically, the aim of this project is to develop technology to kill pests with blue laser beams, to develop new control technologies through breeding superior natural enemy strains and controlling their behavior, and to combine these technologies to establish pest control systems that do not rely on chemical pesticides. Genome sequencing is indispensable to breeding and behavior control research, and I used the research budget to purchase a Hitachi High-Tech DS3000 Compact Capillary Electrophoresis Sequencer, which we are making good use of."

Breeding is mainly carried out by collaborating researchers from NARO (National Agriculture and Food Research Organization), who are working with the cutting-edge breeding method of DNA marker-assisted breeding.[*1]

"The natural enemies are released outside, which means that genetic modification is regulated, and genome editing may also be subject to regulation in some cases. We are therefore aiming to use techniques such as genome editing and RNAi[*2] to efficiently discover genes that are linked to superior performance and to establish natural mating patterns to produce populations that carry these genes," says Prof. Hinomoto.

*1 DNA marker-assisted breeding
A method of breeding in which selection is carried out using base sequences (DNA markers) that are located near useful genes. In conventional breeding, it was only possible to tell if the individuals born of a particular cross had a specific trait by actually rearing them. With DNA marker-assisted breeding, as long as the gene for the desired trait is known, the trait can be selected for at an early stage according to the presence or otherwise of the DNA marker. This greatly increases the efficiency of breeding.

*2 RNAi
This stands for RNA interference, a knockdown method to suppress the target gene. It makes use of the gene regulation mechanism whereby mRNAs with complementary sequences are broken down specifically by double-stranded RNA.

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Understanding natural enemy strains though genome analysis

Prof. Hinomoto's main area of research is behavioral control of natural enemy organisms. He is working to understand the behavioral characteristics of natural enemies in order to establish ways to attract native natural enemies living in the vicinity of agricultural land and to ensure that natural enemy materials that are released on agricultural land stay there.

"When we talk about chemical substances to attract organisms, most people probably think of sex pheromones," he says. "However, I am focusing on herbivory-induced plant volatiles, or HIPVs. These are volatile substances that are released by plants when they are damaged by plant-feeding pests, and they have the property of attracting the natural enemies of these pests. HIPVs were discovered in the 1980s, but little progress was made in clarifying them as they comprise a range of different substances and their composition varies between the species and variety of plant. It is only in recent years that researchers have begun to understand the genes involved in sensing volatile substances and the relationship between sensing these substances and behavior. I believe that if we could use this information to develop a chemical agent that attracts natural enemies, it would be possible to attract native natural enemies to agricultural land from surrounding areas and induce them to stay."

For this to work, it is necessary to know where the natural enemies are usually found—the methods used to bring them in will differ greatly depending on whether they live in the weeds surrounding the agricultural land or in remote mountain forests. "If they live nearby, we have to think of how to attract them; if they live far away, we need to think more in terms of how to make them stay if they come. I am working on genomic analyses of natural enemies in agricultural land and the surrounding areas in order to elucidate genetic lineages and gain an understanding of their migration and distribution. For this, I use simple short repeat sequences called microsatellites. Studying these sequences makes it possible to uncover phyletic relationships within species and blood relationships within populations."

Highly sensitive fragment analysis yields rich results

The recent spread of DNA sequencers has led to the use of genome information in a wide range of fields. However, ecology studies, and in particular the field of pest control, have been slow to use this information, largely because of the time and effort required for stable breeding and reproduction of pests and their natural enemies in the laboratory. Prof. Hinomoto is blazing a trail by combining ecology with the latest findings from molecular biology.

"To be able to use genome information, it is essential to have a DNA sequencer available for use in the laboratory whenever it is needed," Prof. Hinomoto points out. "It is expensive and time-consuming to outsource everything, and in my laboratory in particular we need to analyze a lot of DNA fragments that external providers can't really handle. This means that the DS3000, which has fragment analysis built in as the default application, is absolutely invaluable. All the consumables are in cartridge form so they can be replaced easily and with no wastage, and maintenance is very

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straightforward. This makes the device very user-friendly, even for students."

As well as a means of gaining an understanding of natural enemy distributions, fragment analysis is also used in Prof. Hinomoto's laboratory to investigate the feeding characteristics of natural enemy organisms. Answering questions such as whether natural enemies are eating the target pest on the agricultural land, or what they eat when the pest is not available, will provide important data for behavioral control.

"If you do a complete genome analysis of phytoseiid mites collected from agricultural land, the DNA from the content of their digestive systems will also be detected. So if you wanted to investigate whether the phytoseiid mites are eating a particular pest, you put a marker on the DNA of the pest and then see whether the same marker turns up in the analysis results for the phytoseiid mites. You could run a PCR with specific primers for the pest DNA, but the detection efficiency will be extremely poor if the pest DNA has been broken down by digestion. Fragment analysis using fluorescent primers is highly sensitive and can find differences of just a single base—this means that if the pest that was eaten was a thrips, it can be identified not just at the level of the order, but right down to the individual species. As the organisms we are looking at are not your normal research animals such as mice, markers have to be developed for fragments, and this involves trial and error. However, if you have a sequencer available, you can carry out various different trials, and we certainly appreciate the freedom this device gives us to run tests at different concentrations."

There have been cases where highly sensitive fragment analysis has provided scientific evidence for phenomena that had only been inferred empirically. For example, phytoseiid mites were believed to eat pollen when the pests they normally prey on are not available, and this had been confirmed under breeding conditions, but the proof came when plant genes were found in fragment analysis of phytoseiid mites collected from agricultural land.

"They may be using the proteins in pollen to stay alive. This suggests that it is highly likely that provided there are flowers in bloom, the natural enemies will stay on agricultural land even if there are no pests there. Phytoseiid mites are tiny, about 0.5 mm long, so going around counting them with the naked eye is not a realistic proposition. However, it is obvious from a single glance whether flowers are blooming or not, so that would give you a rough guide as to whether or not there are any natural enemies on the agricultural land."

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Increasing diversity and reducing labor in agricultural land

Prof. Hinomoto believes the concept of integrated pest management (IPM) will be increasingly important in agriculture. This is the approach of combining diverse methods to manage pests and weeds in a rational manner, rather than the conventional, symptomatic method of clearing pests and weeds with chemical pesticides.

“Making use of the fact that natural enemies eat pollen, trials are underway in which flowering plants other than the desired crop are planted. These are called insectary plants. Marigolds, for example, produce a great deal of pollen and they are also home to marigold-loving thrip species. This means that marigolds are effective at attracting native natural enemies that feed on thrips, and also at ensuring that native or commercial natural enemies become established. Some agricultural areas have succeeded in reducing their use of pesticides by planting marigolds around eggplant fields, and research is currently looking into creating environments in which there are always flowers in bloom by combining multiple species of insectary plants that have different flowering periods.”

Modern agriculture has aimed for efficiency through uniform environments in which only the desired crops grow on agricultural land, but the downside is that environments with little diversity are vulnerable to outside threats such as pests. Increasing the diversity of fields themselves, including both plants and animals, is likely to maintain a better balance between organisms so that the entire ecosystem of the agricultural area can become stable.

“Agricultural land in Japan originally had high diversity, and I believe we can regain this diversity through reducing pesticide use. For this, agriculture needs to be considered at the overall regional level, rather than in terms of individual producers. It is important to uncover relationships and interactions between pests and their surrounding environment, which includes their natural enemies and plants, on a regional level.” Prof. Hinomoto has a dream for the future: “Ultimately, I want to bring about pest management where it is enough to plant crops and insectary plants and then introduce biological control agents or take measures to attract native natural enemies, after which it can all just be left alone.” His research started from the desire to do something about the pests that plague the producers of the crops that we need for our survival, and he is determined to develop it further: “I’m aiming for an ideal model of sustainable agriculture that reduces the workload of the producers and enriches the environment.”

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(Interview by Akiko Seki, photos by Yuki Akiyama)

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Analyzing Genetic Alterations in Bone and Soft-Tissue Tumors to Improve Diagnostic Accuracy and Treatment Strategies

Investigators at the Graduate School of Medical Sciences of Kyushu University are working to characterize diseases and improve diagnostic procedures and treatments with the goal of providing optimal care for patients. As part of this work, the graduate school's Department of Anatomic Pathology is studying bone and soft-tissue tumors. Malignant bone and soft-tissue tumors are called sarcomas. Due to their rarity and diverse histological features, investigating their genetic alterations is a key element in the development of pathological diagnostic procedures and treatments. We interviewed Associate Professor Takeshi Iwasaki of the Department of Anatomic Pathology, who conducts experiments and extracts data with the goal of developing accurate diagnostic procedures and appropriate treatments.

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Rare and diverse

Bone and soft-tissue tumors generally refer to tumors of mesenchymal tissues such as bone, muscle, adipose tissue, nerves, and blood vessels. To most people, the terms "cancer" and "tumor" are typically associated with epithelial cancers such as colon and gastric cancer, which differ significantly from bone and soft-tissue tumors in their characteristics.

Associate Professor Takeshi Iwasaki, who specializes in bone and soft-tissue tumors in the Department of Anatomic Pathology of Kyushu University's Graduate School of Medical Sciences, notes, "Colon cancer is often diagnosed based only on microscopy of affected tissue, but bone and soft-tissue tumors are notoriously difficult to diagnose accurately." Their diverse histological features mean that they can rarely be accurately classified based on morphology alone. Their rarity means that few patients are encountered in typical hospitals and that there are few specialists.

Treatment is also challenging

Bone and soft-tissue tumors are also difficult to treat. Gastric cancer and colon cancer are often found at regular physical examinations. In contrast, bone and soft-tissue tumors are typically discovered incidentally when patients seek medical attention for subjective symptoms such as a lump or swelling, often in the foot. Retroperitoneal tumors, which occur deep within the body, often remain undetected until they have grown substantially.

"Given the rare nature of this type of tumor, delayed diagnosis or discovery only after a sarcoma has become quite large may require resection of a larger area and reconstructive surgery (myocutaneous flaps or skin grafts) by a plastic surgeon. Tumors that are already unresectable when they are discovered often carry a poor prognosis."

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Bone and soft-tissue tumors affect everyone from children to elderly people and characteristically occur in many different locations such as the subcutis, muscles, and blood vessels. The recent advent of molecular targeted drugs has brought cancer treatment to a new level. Therapies targeted against specific molecular alterations are now widely used to treat breast cancer and colon cancer, for example.

"But since there is such a wide range of bone and soft-tissue tumors, treatments specific to the many different types have yet to be established."

Although recent research findings have helped physicians better classify diseases based on genetic alterations, many bone and soft-tissue tumors still remain difficult to classify. A growing number of previously unknown genetic alterations are being discovered in this class of tumors, which still remains incompletely studied. As such, few tumor-specific molecular targeted drugs are available.

Seeking optimal genetic analysis procedures

Faced with these challenges, Associate Professor Iwasaki is studying molecular alterations in bone and soft-tissue tumors to inform the development of new diagnostic procedures and treatments.

Immunostaining is commonly used in cancer research. The technique is well suited for characterizing protein expression but cannot be used for analysis of genetic alterations. Gene analysis is instead done with techniques such as gene panel testing and whole RNA sequencing with next-generation sequencers (NGSs). Associate Professor Iwasaki, however, says that these techniques do not suit all research needs.

"I try to establish procedures that enable more accurate diagnosis using FISH, RT-PCR, Sanger sequencing, MLPA, NGS, and other methodologies, but each has its pros and cons in terms of cost, speed, and usability. You have to pick a technique that fits what you want to analyze."

Genetic analyses are typically outsourced to central university laboratories. Associate Professor Iwasaki, however, often needs to quickly analyze only certain genetic alterations at his own pace and therefore finds such arrangements inconvenient.

Another hurdle is sample degradation.

"Kyushu University Hospital is a hub for bone and soft-tissue tumor care and research in the Kyushu area and one of just a few such institutions nationwide. Here in the Department of Anatomic Pathology we have many archived specimens and receive many other specimens from our research partners. But given the rarity of bone and soft-tissue tumors, we are sometimes forced to use specimens that are three decades old. If the nucleic acids are highly fragmented, this can sometimes prevent successful analysis using next-generation sequencing (NGS). As we were seeking to resolve this issue, we were told about the DS3000, a compact capillary sequencer.

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Rapid on-site analysis of specific genetic alterations

Associate Professor Iwasaki tells us that excellent operability and favorable cost performance were behind his team's decision to purchase the DS3000.

"We were convinced once we saw how easy it was to use and how it facilitated our research. DS3000 consumables, moreover, do not come in sets. They can be replenished individually as needed. Replenishment is simple and waste-free because consumables are provided in individual cartridges. This flexible design makes maintenance easy and reduces costs. Finally, Hitachi's extensive experience developing capillary sequencers was also reassuring."

Associate Professor Iwasaki tells us that the laboratory technicians who actually use the instrument report no major inconveniences.

"It initially took effort to get used to operating the instrument, but it's easy to use now. The instrument displays waveform data that closely resembles raw data, which is different from the instrument we previously used. Initially, interpreting the data took time."

Associate Professor Iwasaki, however, says his team overcame this issue by using the other instrument to check data.

"We were grateful to have the opportunity to evaluate the operability and performance of the instrument during a demo period before we purchased the DS3000 in December 2024."

Associate Professor Iwasaki says that the extended demo period they had gave them the opportunity to assess both the advantages and limitations of the instrument that would not have been apparent over a shorter time. This ultimately led to their decision to buy it.

"Scientific instruments are used for a long time and must be chosen carefully. Being able to fully evaluate the instrument during the demo period gave us peace of mind when it came time to buy it."

Associate Professor Iwasaki finds the compact size of the DS3000 appealing.

"Our laboratory is crowded with equipment. We didn't have the space for a big instrument. The small DS3000 fit on a lab bench with room to spare. Since we will be getting more equipment, this compactness is a great feature."

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Fragmented DNA can be analyzed

As stated previously, the DNA in formalin-fixed paraffin-embedded specimens used in research is often fragmented and can be difficult to analyze. Associate Professor Iwasaki's team sometimes analyzed DNA with NGS but was often unsuccessful with library creation.

"In such cases, the DS3000 capillary sequencer was often sufficient to yield usable results. This proved extremely useful."

The team sometimes verifies NGS data with Sanger sequencing, which is very reliable and well suited for verifying the presence of specific mutations.

"Faced with budget constraints, we appropriately choose between NGS techniques and capillary sequencing to get reliable data while keeping costs down. In terms of the balance between research speed and cost, I'm convinced that capillary sequencers will remain indispensable. Capillary sequencers excel at delivering rapid results."

Establishing more accurate diagnostic procedures

"Now that we have the capillary sequencer, we can analyze samples in our laboratory without outsourcing. This arrangement places fewer time constraints on our research and lets us work more freely. When we outsourced to the central university laboratory here we had to work to meet submission deadlines and spend time arranging for shipping. But now, we just load the sample and then check the results on the following day. Since we're able to reliably analyze short target regions with no increase in workload, we have become more efficient."

Many unknown targets remain concealed within bone and soft-tissue tumors. At the same time, advances in sequencing technology have made more analytical techniques available, more capable investigators are coming of age, and costs are falling. Offering quick, inexpensive analyses, capillary sequencers will remain an effective tool. They offer broad capabilities beyond mere mutation analysis, such as fusion gene detection, verification of vector construction, MLPA-based methylation analysis, and copy number analysis. The scope of their use will continue to expand.

"Previous research mainly relied on bulk analysis using techniques such as Sanger sequencing or multiplex analysis, but single-cell analytical methods are now evolving rapidly. We are able to obtain data in finer detail now that analysis is possible on the single-cell level. These strides forward in technology are moving bone and soft-tissue tumor research into a new phase. We will continue with our research using a wide array of analytical tools as we seek to discover more effective treatments in this still understudied field.

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Kyushu University, which has studied anatomical and molecular pathology for over a century, has 160 rare specimens on display at the Kyushu University Museum of Human Anatomy and Pathology on campus. Also on display are historic anatomical models, vintage microscopes, and other medical history artifacts not seen in a typical laboratory setting.

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