

Commentary

Open Access

Commentary: Liquid-phase ASEM imaging of cellular and structural details in cartilage and bone formed during endochondral ossification: Keap1-deficient osteomalacia

Eiko Sakai^{1*}, Mari Sato², Chikara Sato^{2,3,4}

¹Division of Dental Pharmacology, Department of Developmental and Reconstructive Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

²National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan

³School of Integrative and Global Majors (SIGMA), University of Tsukuba, Tsukuba, Japan

⁴Biological Science Course, Graduate School of Science and Engineering, Aoyama Gakuin University, Sagami-hara, Japan

Article Info

Article Notes

Received: October 22, 2022

Accepted: December 06, 2022

*Correspondence:

*Dr. Eiko Sakai, Division of Dental Pharmacology, Department of Developmental and Reconstructive Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; Email: eiko-s@nagasaki-u.ac.jp

©2022 Sakai E. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License.

Keywords

Liquid-phase

Atmospheric scanning electron microscopy

Endochondral ossification

Cartilage

Keap1

Osteomalacia

The development of microscopies, to observe microstructures as they are, has been achieved through the accumulations of efforts of numerous motivated scientists and engineers. Clear images of microstructures in tissues and cells provide insights into their mechanism and improve our understanding of the tissue.

Atmospheric scanning electron microscopy (ASEM), developed by Chikara Sato of Advanced Industrial Science and Technology (AIST) and scientists in Japan Electron Optics Laboratory (JEOL) in 2010¹, is a unique inverted SEM that enables the observation of tissues and cells immersed in aqueous solutions, without the requirement of drying the samples. This enables the observation of tissues containing water in a stable aqueous buffer environment.

During mammalian skeletal development, bone elongation occurs at both ends via the replacement of cartilage with mineralized bone, in a process called endochondral ossification. This process is facilitated by the formation of new blood vessels, as well as interactions between the cartilage and bone cells including osteoclasts, osteoblasts, and osteocytes. However, the origin of osteoblasts is unclear and the process of the transformation of chondrocytes into osteoblasts is controversial. These open questions have been posed to understand the mechanisms underlying endochondral ossification. To address them, ASEM is expected to be useful because cartilage tissues contain a large amount of water.

In our recent article, we imaged thick sections of cartilage tissues at various stages in the development using ASEM². Our results represent the first liquid-phase electron microscopy (EM) observations of endochondral ossification. The purpose of this commentary is to provide information about the ASEM and how we successfully used ASEM for the observation of hard tissue immersed in liquid, as well as the methodology employed, obtained results, and the prospects.

An ASEM dish is a standard plastic Petri dish-shaped sample holder of 35-mm diameter, with eight windows, each measuring 250 × 250 μm² in length and width, of an ultra-thin silicon nitride (SiN) film, approximately 100 nm thick. These windows in the bottom isolates the specimen from the underneath vacuum. Electron beams pass through the SiN film, which allows aldehyde-fixed cells and tissues to be observed in aqueous solutions. To minimize the effect

of radicals formed by electron radiation at low-accelerating voltage (30kV), the observation buffer contained radical scavenger glucose (10 mg/mL w/v d-glucose in 1 mM cacodylate buffer (pH 7.4) and 60 mM KCl). The close contact between the specimen and window is crucial for capturing clear images.

When unstained hard tissues were imaged using ASEM, areas rich in minerals were observed as bright signals. Therefore, calcified areas can be visualized without staining. After staining with 2% phosphotungstic acid (PTA) solution, proteins and nucleic acids of the specimen are brightly imaged, which facilitates visualization of non-calcified cells and connective tissues. ASEM enabled the observation of the blood cells flowing through the blood vessels while preserving the structure of the water- and mucus- filled blood vessels, and enabled us to image the blood vessels invading the periosteum from outside the bone. Haematopoietic cells protruding from the blood vessels invading the perichondrium could be observed while preserving the tube-like structure and without collapsing the blood vessels. Additionally, the images obtained give stereoscopic-like impression as observable specimen thickness is 2–3 μm from the SiN film: structures near the SiN film imaged sharply while image get blurred as the structure get apart from the film³.

Using these methods, we succeeded in observing the endochondral ossification process in mice on embryonic day 15.5, postnatal day 1, postnatal day 6, and postnatal day 10. The aldehyde-fixed femurs and tibias including the joints of the mice, were embedded in 4% agar, sliced into thick slices (200 μm) using a linear slicer, and placed on a window for observation. The mineralized area of the cancellous bone region was observed before and after staining with PTA. We found it easy to slide the same sample on the SiN film window slightly to increase observation areas.

Kelch-like ECH-associated protein 1 (Keap1) is a negative regulator of nuclear factor E2 p45-related factor 2 (Nrf2), which upregulates phase 2 antioxidative stress enzymes. Keap1-deficient mice, in which Nrf2 is constantly activated, exhibit reduced oxidative stress levels⁴. We tested the efficacy of liquid-phase EM in observing the epiphyseal cartilage of 6-day postnatal wild-type and *Keap1* gene-deficient mice. Only after aldehyde fixation and staining without further additional steps, we acquired images of impaired calcification (osteomalacia) and angiogenesis of the epiphyseal cartilage in *Keap1*-deficient mice using ASEM². Quick ASEM appears to be well-suited for observing cartilage, especially in neonatal mice, in which bones are sufficiently soft and easy to slice. The above observations show the time-saving potential of ASEM to greatly accelerate bone research.

Furthermore, as shown in a previous paper⁵, immune-gold staining can be performed using ASEM, even though this method involves the scanning electron microscopy. However, to prevent non-specific bindings of antibodies, adequate blocking condition had to be found for the antibody. In addition, the dish-shaped specimen holder allows the primary cell culture on it. After fixation, the cells immersed in the observation buffer on the dish can be directly imaged using ASEM.

The ASEM method used in this study is a valuable analytical tool that can be used to further our understanding of the development of mammalian bone tissue. Additionally, this technique could help future clinical studies aimed at understanding the effect of trauma, disease, and surgery on the healing process. Bone healing is a multifaceted and complex process that requires angiogenesis and mechanical stability, along with osteogenesis and osteoinduction. For the induction of bone regeneration, autogenous bone grafts or bone substitute procedures have been used in traumatology, tumor surgery, and revision arthroplasty. Currently, the observation of bone graft or bone substitute and its surrounding tissue is extremely challenging during bone regeneration in vivo; however, in this study, ASEM has demonstrated the potential for observation of osseointegration, as well as assessment of osteoconductivity. Recently, osteoinductive materials, such as demineralized bone matrix and bone morphogenetic proteins, have garnered increasing attention. ASEM can be used in in vitro comparative studies of the bone regeneration ability of these osteoinductive materials. The ASEM sample holder is a removable dish, enabling cells culturing in a CO₂ incubator. We previously performed primary culture of calvaria-derived osteoblasts on an ASEM dish, and succeeded in observing bright signals⁴. Elemental composition analysis using the energy dispersive X-ray spectrometer showed that the bright signals in ASEM images show calcium phosphate (CaP) mineralization. Through dynamic monitoring of non-organic CaP crystal growth related to osteoinductive materials in an ASEM dish, ASEM technology contributes to the development of ideal materials for artificial bone. In cultured autologous chondrocyte implantation, a small amount of a patient's normal cartilage cells is collected, and the cartilage cells are mixed with matrix, such as atelocollagen, and further cultured outside the body to form cartilage tissue. In such clinical treatment, the high-throughput and rapid ASEM imaging of wet tissue at EM resolution may prove to be an extremely effective tool to check samples.

Conclusion

ASEM technology allows hard tissue immersed in aqueous liquid to be imaged at EM resolution under atmospheric pressure. ASEM has the potential for use not only in basic research, but also in clinical research, including those on

bone healing, bone regeneration, and autologous cultured chondrocyte implantation. The rapid, high-throughput ASEM method could be a powerful tool for understanding the detailed regulation mechanisms of hard tissues.

Conflict of Interest

The authors have no relevant conflict of interest.

Funding

No funding has been received for this article.

References

1. Nishiyama H, Sato C, Ogura T, et al. Atmospheric Scanning Electron Microscope Observes Cells and Tissues in Open Medium Through Silicon Nitride Film. *J Struct Biol.* 2010; 169: 438-449.
2. Sakai E, Tsukuba T, Sato C, et al. Liquid-Phase ASEM Imaging of Cellular and Structural Details in Cartilage and Bone Formed during Endochondral Ossification: Keap1-Deficient Osteomalacia. *Sci Rep.* 2021; 11: 1-17.
3. Maruyama Y, Ebihara T, Nishiyama H, et al. Immuno EM-OM correlative microscopy in solution by atmospheric scanning electron microscopy (ASEM). *J Struct Biol.* 2012; 180: 259-70.
4. Sakai E, Morita M, Tsukuba T, et al. Effects of Deficiency of Kelch-Like ECH-Associated Protein 1 on Skeletal Organization: a mechanism for Diminished Nuclear Factor of Activated T Cells Cytoplasmic 1 During Osteoclastogenesis. *FASEB J.* 2017; 31: 4011-4022.
5. Sato C, Yamazaki D, Sakai E, et al. Calcium phosphate mineralization in Bone Tissues Directly Observed in Aqueous Liquid by Atmospheric SEM (ASEM) Without Staining: Microfluidics Crystallization Chamber and Immune-EM. *Sci Rep.* 2019; 9: 1-13.