

ORIGINAL RESEARCH

Three-dimensional fine structures of the maculae flavae of the human vocal fold using correlative light and electron microscopy

Kiminobu Sato MD¹  | Takashi Kurita MD¹ | Fumihiko Sato MD¹  |
Keisuke Ohta PhD² | Kiminori Sato MD¹  | Shun-ichi Chitose MD¹  |
Takeharu Ono MD¹  | Hirohito Umeno MD¹ 

¹Department of Otolaryngology-Head and Neck Surgery, Kurume University School of Medicine, Kurume, Japan

²Advanced Imaging Research Center, Kurume University School of Medicine, Kurume, Japan

Correspondence

Kiminobu Sato, Department of Otolaryngology-Head and Neck Surgery, Kurume University School of Medicine, Kurume 830-0011, Japan.
Email: satou_kiminobu@med.kurume-u.ac.jp

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Abstract

Objectives: To analyze various aspects of complex tissue, there is increasing demand to study each sample at different length scales in biology. Correlative light and electron microscopy (CLEM) is the latest technique to correlate two different types of information on the exact same histological area of interest: histology (from light microscopy) and ultrastructure (from electron microscopy). The three-dimensional fine structures of the maculae flavae (MFe) of the human vocal fold were investigated using CLEM.

Methods: Five normal human adult vocal folds as specimens embedded in paraffin, sectioned, and mounted on glass slides with/without a chemical digestion method (modified sodium hydroxide maceration method) were investigated. Observations using CLEM were performed.

Results: The fine structures of cells and extracellular matrices in the MFe and their peripheral regions were able to be observed on the exact same histological area of interest with the light microscope and field emission-scanning electron microscope. Cobblestone-like polygonal cells, vocal fold stellate cell-like cells, and fibroblast-like spindle cells were intermingled in the MFe of the human vocal fold. The extracellular matrices surrounding each three types of cell in the MFe differed, suggesting the cells were different in functional property.

Conclusion: CLEM is a useful technique to observe the three-dimensional fine structures of the human vocal fold mucosa. The results of the present study are consistent with the hypothesis that the cells in the MFe of the human vocal fold have heterogeneity and each three types of cell have different properties.

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KEYWORDS

correlative light and electron microscopy, human vocal fold, maculae flavae, three-dimensional fine structures

1 | INTRODUCTION

Biological microscopy including light microscopy and electron microscopy is a standard method to observe cells and tissue structures. To elucidate three-dimensional fine structures of the cells and extracellular matrices provides evidence of the functional activities.

Light microscopy shows structures of tissues, on the other hand, scanning electron microscopy (SEM) provides images of three-dimensional fine structures of the cells and tissues. However, the subject area of observation under electron microscopy is limited as compared with light microscopy and it is difficult to observe limited areas or transition areas in complex tissues under electron microscopy. To analyze various aspects of complex tissue, there is increasing demand to study each single sample at different length scales in biology.

Correlative light and electron microscopy (CLEM) is the latest technique to correlate two different types of information using light and electron microscopy on the exact same histological area of interest.¹⁻³

Fine structures of the human vocal fold mucosa using electron microscopy have been reported in our previous studies.⁴⁻⁹ The results of these studies were obtained by cultivating the technique of collecting samples from the histological area of interest in the human vocal fold mucosa. However, the technique required skill to prepare the very small specimens from the human vocal fold mucosa for electron microscopy.

The previous studies revealed there is growing evidence that the maculae flavae (MFe) are a stem cell niche containing tissue stem cells of the human vocal fold mucosa.⁸⁻¹³ Recent studies have suggested that there are three phenotypes of cells (cobblestone-like polygonal cells, vocal fold stellate cell-like cells, and fibroblast-like spindle cells) in the MFe of the human vocal fold mucosa and these cells have heterogeneity and hierarchy in the stem cell system *in vivo*.^{14,15}

The human larynx has a complex structural organization with a framework characterized by an external cartilaginous skeleton and internal connective tissue. And the framework composed of cartilage, ligaments, and muscles contributes to the physiologic functions of the human larynx.¹⁶

The hypothesis of the study is to determine whether distinct information from light and electron microscopy can be obtained through CLEM that may be of use in further study of the MFe of the vocal fold.

This study investigated the fine structures of cells and extracellular matrices in the MFe of the human vocal fold mucosa using CLEM.

2 | MATERIALS AND METHODS

2.1 | Sample preparation

All experiments were performed with the approval of the Ethical Committee of Kurume University (permit number: 21129) and informed consent from participants was received. Normal five human

adult larynges obtained from autopsy or surgical cases (38–83 years old, two males and three females) were investigated.

Any lesions or diseases that affect the tissue of the vocal fold were not included.

2.2 | Light microscopy

Human larynges were fixed in 10% neutral formalin, dehydrated in graded concentrations of ethanol, embedded in paraffin, and sectioned to a thickness of 4 μm . The specimens were mounted onto glass slides. All specimens were stained with Hematoxylin & Eosin stain (Sakura Finetek Japan Co., Ltd.) and observed under light microscopy.

To observe the three-dimensional structures of collagen, reticular, and elastic fibers, a chemical digestion method (sodium hydroxide maceration method) was performed to digest ground substances. The specimens stained with Hematoxylin & Eosin were immersed in 2 mol/L NaOH (Nacalai Tesque) for 2 h at room temperature.

2.3 | Scanning electron microscopy

The same specimens mounted onto glass slides and stained with Hematoxylin & Eosin were used for SEM. To coat the surface of specimens for SEM, the specimens were immersed in xylene, the cover glasses were removed, and the dry specimens were stained with 0.5% AuCl₃. To produce a thin film on the glass, vacuum evaporation was performed in a carbon coating device (carbon coater CADE-E, Meiwa-fosis). The sections were set on the stage with adhesive conductive tape in a low-vacuum SEM (Quanta 3D FEG, FEI Company). After evacuation of the specimen chamber, the sections were observed under the electron beam with an accelerating voltage of 5 kV.

2.4 | Correlating light and electron microscopy

To correlate the images observed by light microscopy and electron microscopy, each image of light microscopy and electron microscopy was taken in the exact same histological area of interest. Any histological areas including the vocal fold mucosa, MFe, and lamina propria obtained under light microscopy were able to be zoomed in on to observe the fine structures under electron microscopy.

3 | RESULTS

The histological approach described above enabled observation of whole specimens of human larynges mounted onto glass slides with

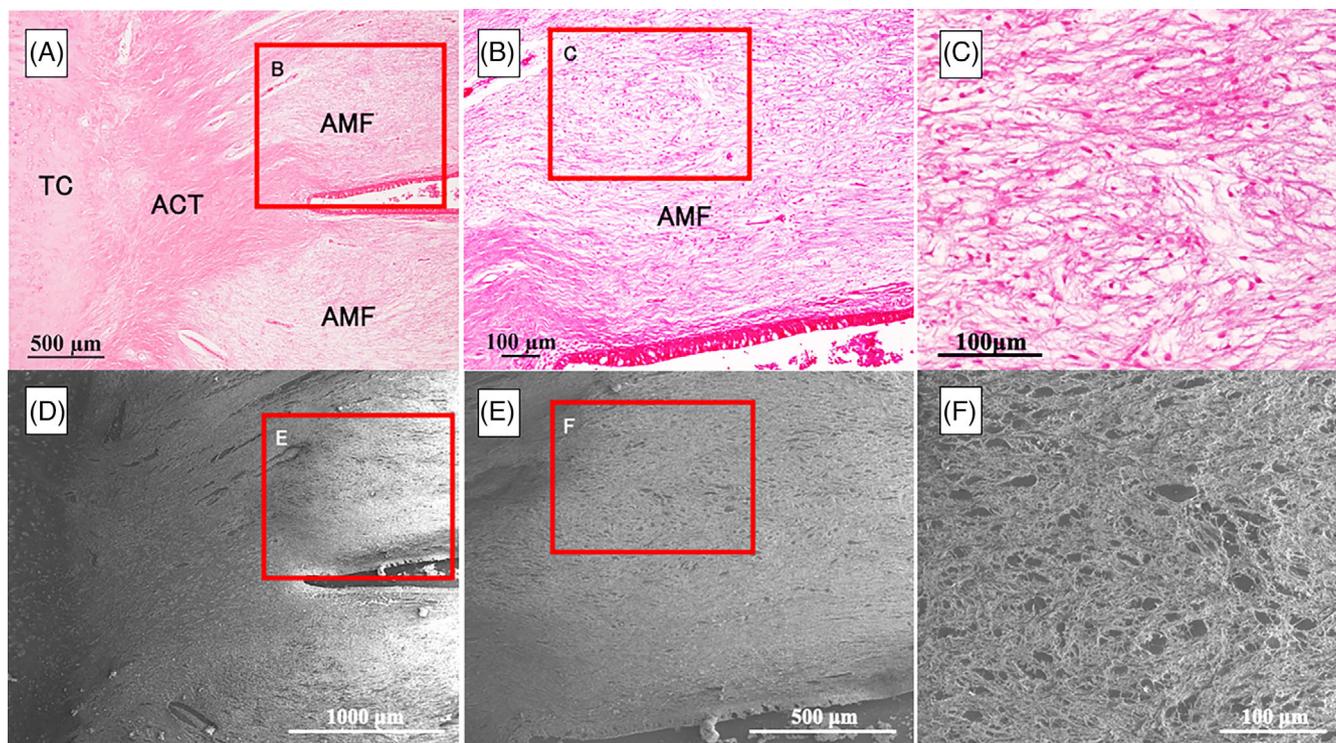


FIGURE 1 Correlative light and electron microscopic images of human laryngeal specimens. (A–C) H&E staining, low magnification of human larynx including thyroid cartilage (TC), anterior commissure tendon (ACT), and anterior macula flava (AMF) (B: square region in (A); (C): square region in (B)). (D–F) SEM, the exact same histological area of interest with light microscopy, and electron microscopy (A correlates with D; B with E; C with F, respectively) (E: square region in D; F: square region in E).

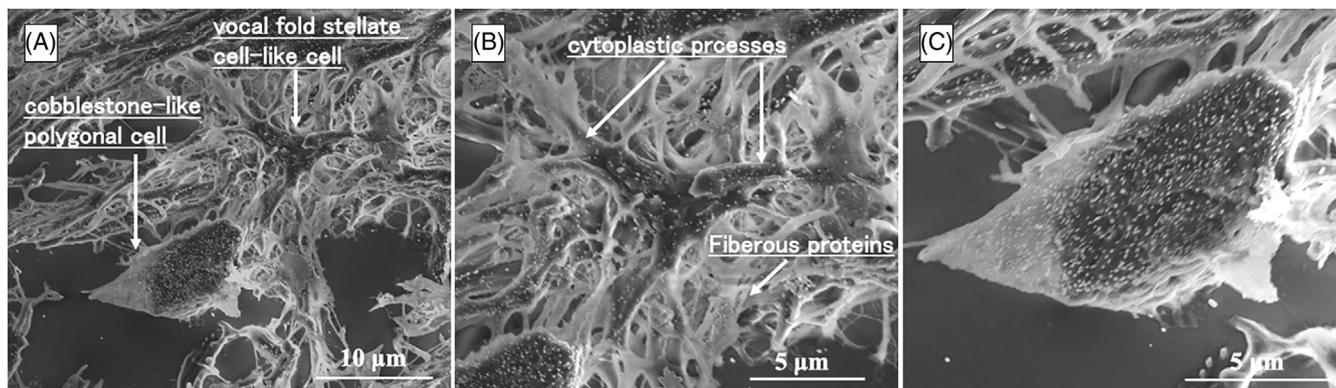


FIGURE 2 High-magnification images of SEM in the AMF. (A) Vocal fold stellate cell-like cell and cobblestone-like polygonal cell present in the AMF. (B) The vocal fold stellate cell-like cell was irregular and stellate in shape and possessed slender cytoplasmic processes. Many fibers ran in various directions around the vocal fold stellate cell-like cell. (C) Fibers were sparse around the cobblestone-like polygonal cell.

Hematoxylin & Eosin stain under SEM. Two different types of information on the exact same histological area of interest, histology (from light microscopy), and ultrastructure (from electron microscopy), could be correlated using the CLEM technique.

3.1 | Anterior and posterior MFe

Figure 1 shows the specimens around the anterior commissure of the human larynx. The thyroid cartilage, anterior commissure tendon, and

anterior macula flava were observed under light microscopy (Figure 1A–C). The exact same histological areas of interest under light microscope were zoomed in on to observe the fine structures under SEM (Figure 1D–F).

At high magnification of the anterior macula flava, different morphological types of cells including vocal fold stellate cell-like cells and cobblestone-like polygonal cells were present (Figure 2). Vocal fold stellate cell-like cells were irregular and stellate in shape and possessed slender cytoplasmic processes. Many fibers ran in various directions around the vocal fold stellate cell-like cells (Figure 2B). On the

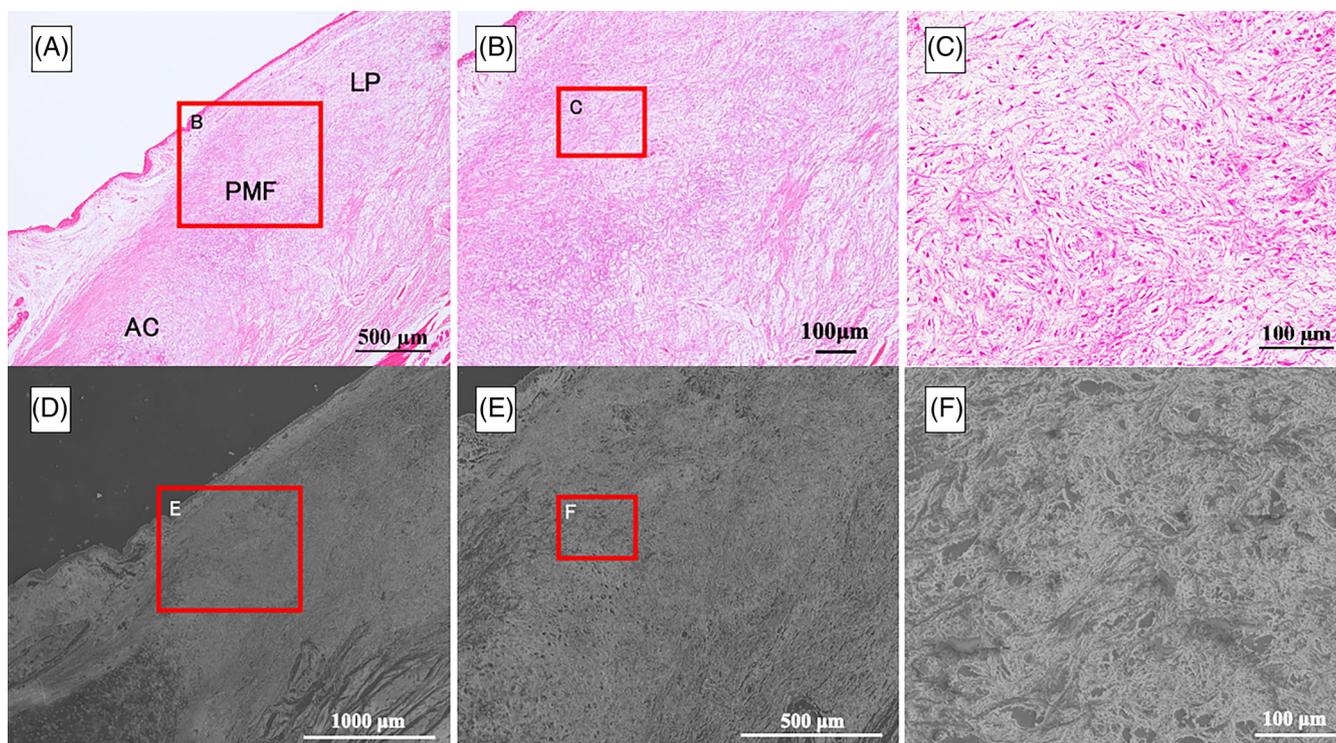


FIGURE 3 Correlative light and electron microscopy of human laryngeal specimens. (A–C) H&E staining, low magnification of human larynx including vocal process of arytenoid cartilage (AC), posterior maculae flava (PMF), and lamina propria of the mucosa (LP) (B: square region in A; C: square region in B). (D–F) SEM, the exact same histological area of interest with light microscopy and electron microscopy (A correlates with D; B with E; C with F, respectively) (E: square region in D; F: square region in E).

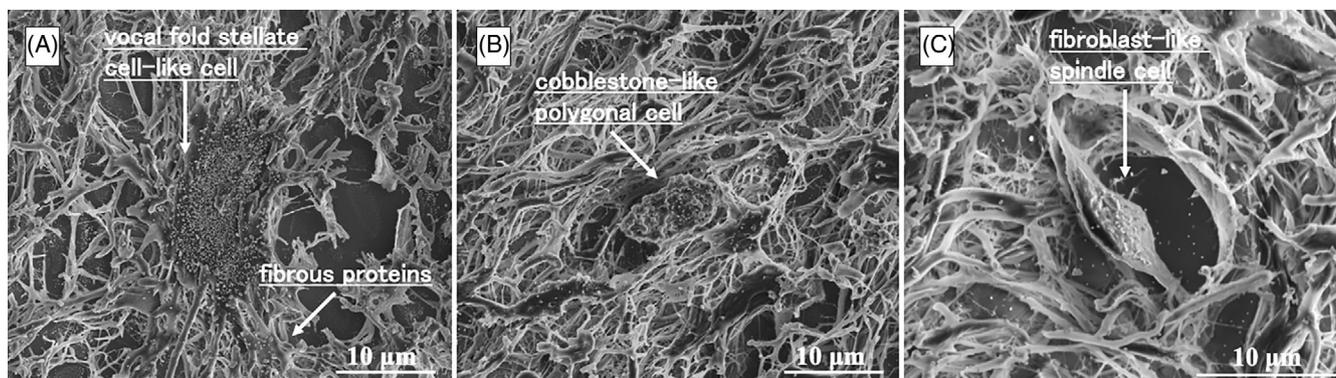


FIGURE 4 High-magnification images of SEM in the PMF. A vocal fold stellate cell-like cell, cobblestone-like polygonal cell, and fibroblasts-like spindle cell were present. (A) The vocal fold stellate cell-like cell was irregular and stellate in shape and possessed slender cytoplasmic processes. Many fibers ran in various directions around the vocal fold stellate cell-like cell. (B) Fibers were sparse around the cobblestone-like polygonal cell. (C) Fibers were sparse around the fibroblast-like spindle cell.

other hand, fibers were sparse around cobblestone-like polygonal cells (Figure 2C).

Figure 3 shows the specimens around tip of the vocal process. The vocal process of the arytenoid cartilage, posterior macula flava, and lamina propria of the mucosa were observed under light microscopy (Figure 3A–C). The exact same histological areas of interest under light microscopy were zoomed in on to observe the fine structures under SEM (Figure 3D–F).

At high magnification of the posterior macula flava, different morphological types of cells including vocal fold stellate cell-like cells, cobblestone-like polygonal cells, and fibroblasts-like spindle cells were present (Figure 4). Many fibers ran in various directions around vocal fold stellate cell-like cells (Figure 4A). On the other hand, fibers were sparse around cobblestone-like polygonal cells and fibroblasts-like spindle cells (Figure 4B,C).

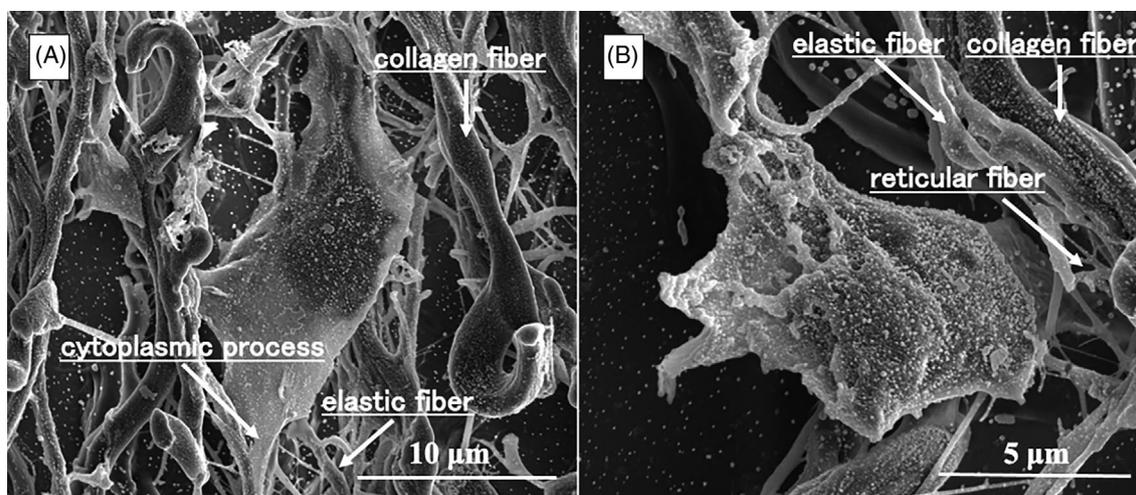


FIGURE 5 High-magnification images of SEM in the AMF using a chemical digestion method. Ground substances around the vocal fold stellate cell-like cell were digested.

Three morphological different types of cells were present in both anterior and posterior MFe. In addition, the extracellular matrices around each three type of cell differed respectively.

High-magnification images of the MFe using a chemical digestion method under SEM are shown in Figure 5. Ground substances around the vocal fold stellate cell-like cells were digested resulting in fibrous proteins such as collagen and elastic fibers which were present around the vocal fold stellate cell-like cells.

3.2 | Transition area between the anterior commissure tendon and anterior macula flava

The specimens of the human vocal fold including the transition area between the anterior macula flava and anterior commissure tendon were observed under light microscopy (Figure 6A,B). The exact same histological areas of interest under light microscopy were zoomed in on to observe the fine structures under SEM (Figure 6C,D). High-magnification images of the transition area between the anterior commissure tendon and anterior macula flava under SEM are shown in Figure 6E–G. The fibrous protein in the anterior commissure tendon was composed of large bundles of collagen fibers (Figure 6F) and the anterior macula flava was composed of elastic fibers and collagen fibers (Figure 6G), respectively. The fiber components of the extracellular matrices abruptly changed in transition area between the anterior commissure tendon and anterior macula flava.

3.3 | Transition area between the anterior maculae flava and lamina propria of vocal fold mucosa

The specimens of the human vocal fold including the transition area between the anterior macula flava and lamina propria were observed under light microscopy (Figure 7A,B). The exact same histological area

of interest under light microscopy was zoomed in on to observe the fine structures under SEM (Figure 7C,D). Fibrous proteins in the lamina propria were composed of elastic, reticular, and collagen fibers (Figure 7E), and the anterior macula flava was composed of elastic, reticular, and collagen fibers (Figure 7F). The fiber components of the extracellular matrices gradually changed in the transition area between the anterior macula flava and lamina propria.

3.4 | Transition area between the posterior maculae flava and lamina propria of vocal fold mucosa

The histological findings using CLEM were the same as those of the transition area between the anterior macula flava and lamina propria of the vocal fold mucosa.

4 | DISCUSSION

4.1 | Morphological evaluation under biological microscopy

Functional morphology involves the study of relationships between the structure and function of an organ. Three-dimensional fine structures of cells and extracellular matrices provide evidence of functional activities. Not only molecular markers and gene expressions but also morphological characteristics of the cells are important in functional morphology. Biological microscopy including light microscopy and electron microscopy is a standard method to observe cells and extracellular matrix structures.

Light microscopy enables researchers to investigate whole sections of specimens. Specimens for light microscopy on paraffin-embedded tissues are generally used for histological research and clinical pathology. In addition, they are inexpensive and easy to prepare

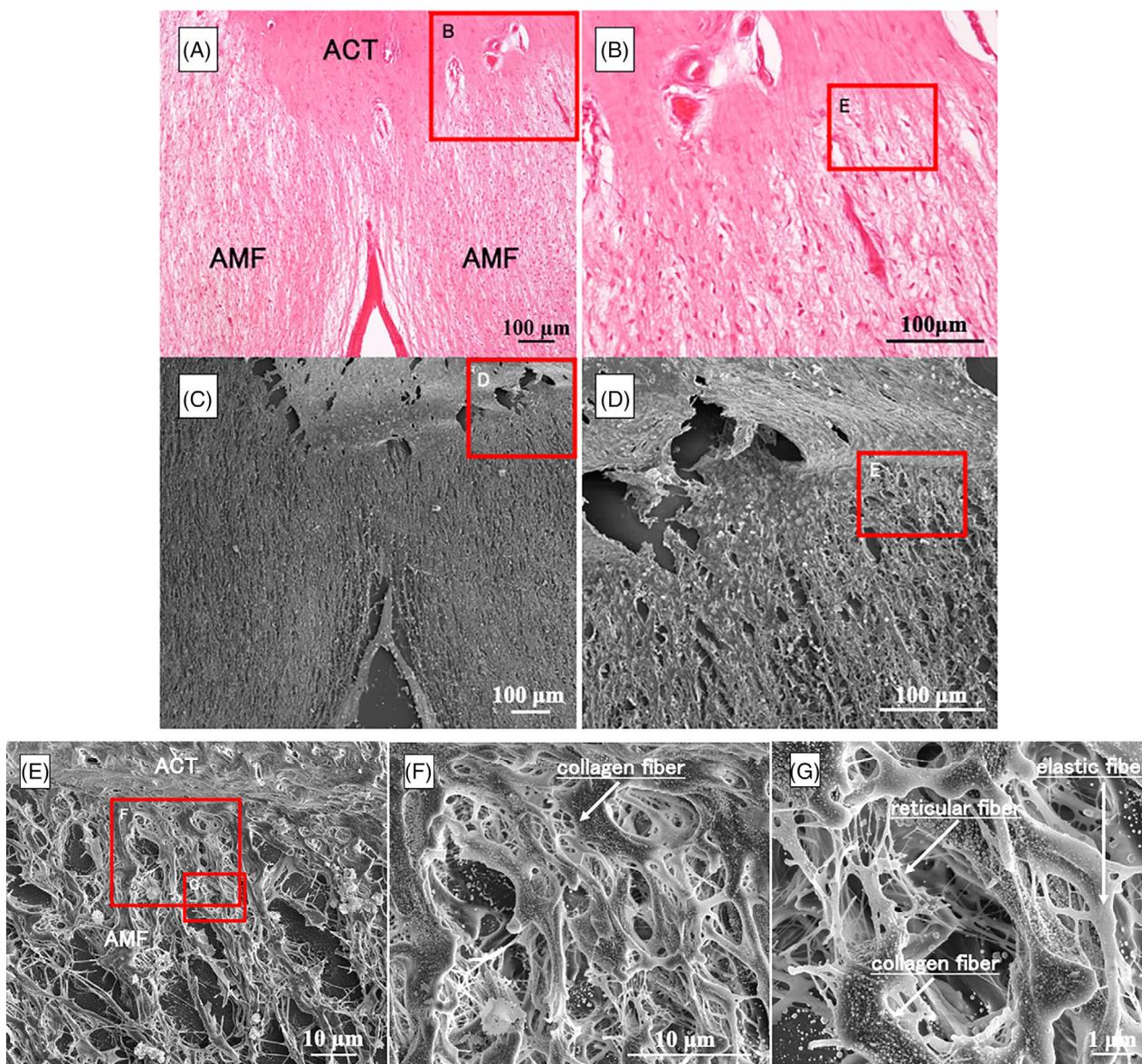


FIGURE 6 Correlative light and electron microscopy of the transition area between AMF and ACT. (A,B) H&E staining, low magnification of human larynx including anterior commissure tendon (ACT) and anterior maculae flava (AMF) (B: square region in A). (C,D) SEM, the exact same histological area of interest with light microscopy and electron microscopy (A correlates with C; B with D, respectively) (D: square region in C). (E–G) High-magnification images of SEM in the ACT and AMF (E: square region in D; F: square region in E; G: square region in E). ACT was composed of large bundles of collagen fibers and the AMF was composed of elastic fibers, reticular fibers, and collagen fibers, respectively. Fiber components of extracellular matrices abruptly changed in transition area between ACT and AMF.

for experiment. However, the images under light microscopy are two-dimensional and restrict researchers' ability to analyze the fine structures of cells and extracellular matrices.

Electron microscopy, especially SEM, enables researchers to investigate the three-dimensional fine structures of cells and extracellular matrices using high-magnification imaging. However, the subject area of observation under electron microscopy is limited as compared with light microscopy. Therefore, it has been challenging to observe subject areas or transition areas in complex tissues like the larynx or vocal fold under electron microscopy. In addition, preparing specimens for electron microscopy is a complex procedure and it requires skill to prepare the very small specimens from tissues.

Recently, a cutting-edge technology, CLEM, allows researchers to investigate cells and extracellular matrices. Low vacuum SEM has a high-sensitivity semiconductor backscattered electron detector with a charge reduction. The imaging of non-conducting specimens under low vacuum SEM allows observation with few charge accumulations on the paraffin sections on glass slides.¹⁷ In the present study, the electron microscopic images with few charge accumulations on glass slides were obtained under low vacuum SEM.

CLEM is the latest technique to correlate two different types of information on the exact same histological area of interest.^{1–3} This technique provides exact structural information by correlating dynamic biological observation with high magnification in the same

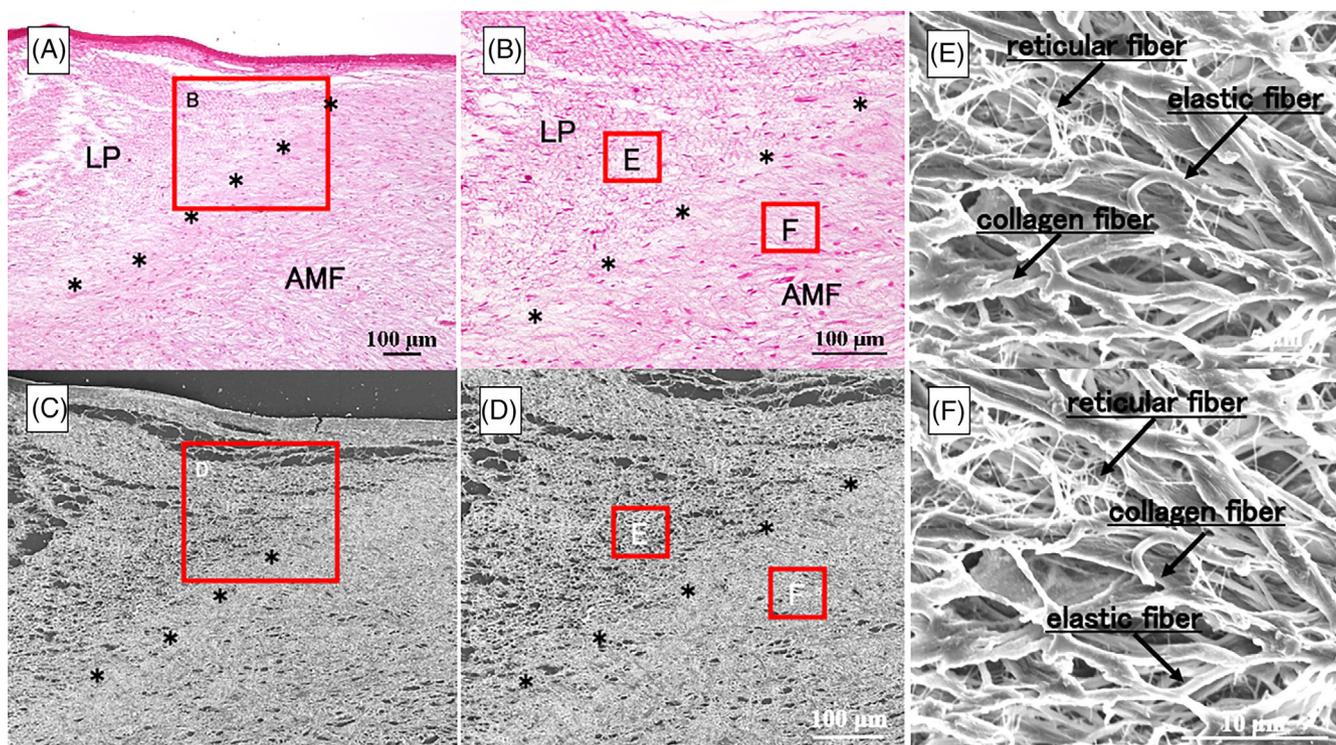


FIGURE 7 Correlative light and electron microscopy of the transition area between AMF and LP (*boundary between AMF and LP). (A,B) H&E staining, low magnification of human larynx including anterior maculae flava (AMF) and lamina propria of the mucosa (LP) (B: square region in A). (C,D) SEM, the exact same histological area of interest with light microscopy and electron microscopy (A correlates with C; B with D, respectively) (D: square region in C). (E) High-magnification image of SEM in LP (square region in B). Elastic, reticular, and collagen fibers were present. (F) High-magnification image of SEM in AMF (square region in B). Elastic, reticular, and collagen fibers were present.

histological area. Electron microscopic images of small isolated areas restrict observation of relevant parts of the system and interactions in the tissues. To analyze various aspects of complex tissue, there is increasing demand to study each single sample at different length scales in many disciplines.

4.2 | Fine structures of the human vocal fold mucosa using CLEM

The human larynx has a complex structural organization with a framework characterized by an external cartilaginous skeleton and internal connective tissues including ligaments, vocal fold mucosae, and muscles.¹⁶ The characteristic structures arranged in the different regions in the larynx contribute to physiologic functions of the human larynx.

Fine structures of the human vocal fold mucosa using electron microscopy have been reported in our previous studies.⁴⁻⁹ These studies revealed the fine structures of the vocal fold mucosa including the MFe of the human adult and newborn larynges. In our previous studies, great skill was required to collect samples from small isolated areas of interest in the human vocal fold mucosa. Therefore, observation techniques of electron microscopy in these studies limited the investigation to confined areas of the vocal fold.

The previous studies revealed that the MFe are dense masses of cells and extracellular matrices and that they are stem cell niches containing tissue stem cells of the human vocal fold mucosa.⁸⁻¹³ In this

study, whole specimens of the anterior and posterior MFe were observed on the exact same histological areas of interest under light and electron microscopy. Extracellular matrices in the macula flava were composed of collagen, elastic and reticular fibers, and ground substances. The present study using CLEM revealed that the components of extracellular matrices gradually changed in the transition area between both the anterior and posterior MFe and the lamina propria of the vocal fold mucosa. On the other hand, the components of extracellular matrices abruptly changed in the transition area between the anterior commissure tendon and anterior macula flava.

Recent studies have shown that there are three phenotypes of cells (cobblestone-like polygonal cells, vocal fold stellate cell-like, cells and fibroblast-like spindle cells) in the macula flava of the human vocal fold mucosa and that these cells have heterogeneity and hierarchy in the stem cell system *in vivo*.^{14,15} In this study, three different morphological types of cells including cobblestone-like polygonal cells, vocal fold stellate cell-like cells, and fibroblast-like spindle cells were observed in the MFe on the exact same histological areas of interest using CLEM. Vocal fold stellate cell-like cells which synthesize extracellular matrices including collagen, reticular and elastic fibers are irregular and stellate in shape and possess slender cytoplasmic processes.⁴ Using CLEM, the present study showed that the vocal fold stellate cell-like cells were surrounded by extracellular matrices including abundant fibrous proteins. On the other hand, two other morphological types of cells were surrounded by deficient fibrous proteins. These results suggested that each type of cell has different functional properties.

4.3 | Application of CLEM to various samples and experimental studies

In this experimental study, paraffin-embedded sections on glass slides were utilized for observations under SEM. Paraffin-embedded specimens are generally used for storing samples and are valuable for the investigation of morphology or histopathology retrospectively. The present CLEM technique is able to observe various paraffin-embedded sections of the larynges under electron microscopy.

CLEM makes it possible to observe small specimens on the exact same histological areas of interest obtained from experimental animals and cultured cell lines.^{2,3} Further applications of CLEM to laryngology would promote elucidation of laryngeal morphology by visualizing the three-dimensional fine structures of the cells and extracellular matrices.

5 | CONCLUSION

CLEM is a powerful technique to observe the three-dimensional fine structures of the human vocal fold mucosa. Fine structures of the human vocal fold mucosa including the anterior and posterior MFe and their peripheral regions were able to be investigated using CLEM. The three-dimensional fine structures under electron microscopy were able to be observed on the exact same histological areas of interest as under light microscopy.

The results of the present study are consistent with the hypothesis that the cells in the MFe of the human vocal fold have heterogeneity and each type of cell has different functional properties.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Kiminobu Sato  <https://orcid.org/0000-0001-6537-4490>

Fumihiko Sato  <https://orcid.org/0000-0002-7990-7590>

Kiminori Sato  <https://orcid.org/0000-0001-5415-0176>

Shun-ichi Chitose  <https://orcid.org/0000-0002-9307-2331>

Takeharu Ono  <https://orcid.org/0000-0003-2414-7034>

Hirohito Umeno  <https://orcid.org/0000-0002-2347-495X>

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