The Laryngoscope
Lippincott Williams & Wilkins

Imaging of the Calf Vocal Fold With High-Frequency Ultrasound

Conor J. Walsh, MSME; James T. Heaton, PhD; James B. Kobler, PhD; Thomas L. Szabo, PhD; Steven M. Zeitels, MD, FACS

Objectives/Hypothesis: High-frequency ultrasound imaging offers the potential for assisting in the diagnosis and treatment of vocal fold pathology if it allows aspects of vocal fold microstructure to be visualized noninvasively. The objective of this study was to assess the ability of high-frequency ultrasound to image vocal fold anatomy and injected biomaterials.

Study Design: The vocal folds of two excised calf larynges were imaged ex vivo and compared with corresponding histological sections.

Methods: High-frequency ultrasound imaging was performed under saline submersion using 40 and 50 MHz transducers, and corresponding cryostat cross-sections were stained with H&E, Trichome, and Verhoeff's Van Gieson stains.

Results: The epithelial surface, lamina propria, and underlying muscle were easily identified with the high-frequency ultrasound as verified with histological sections representing each imaged region. The arytenoid cartilage vocal process can also be clearly distinguished from the surrounding tissue, as can the full extent of injected biomaterials within the superficial lamina propria. Useful ultrasound resolution was obtained to depths of at least 10 mm within the tissue with the 40 MHz transducer.

Conclusions: This preliminary study demonstrates the capability of high-frequency ultrasound to image the layered anatomy of the calf vocal fold and to discern materials injected into the superficial lamina propria, indicating that this technology holds a strong potential for use in phonosurgery.

From the Department of Mechanical Engineering (C.J.W.), Massachusetts Institute of Technology, and Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA, U.S.A.; Department of Surgery, Harvard Medical School (J.T.H., J.B.K., S.M.Z.), Center for Laryngeal Surgery and Voice Rehabilitation, Massachusetts General Hospital, Boston, Massachusetts, U.S.A.; and Department of Biomedical Engineering (T.L.S.), Boston University, Boston, Massachusetts, U.S.A.

Editor's Note: This Manuscript was accepted for publication May 7, 2008.

Supported by the Eugene B. Casey Foundation and the Institute of Laryngology and Voice Restoration.

Send correspondence to James T. Heaton, PhD, Department of Surgery Havard Medical School, Center for Laryngeal Surgery and Voice Rehabilitation, Massachusetts General Hospital, One Bowdoin Square, 11th Floor, Boston, MA 02114. E-mail: james.heaton@mgh.harvard.edu

DOI: 10.1097/MLG.0b013e31817f18ca

Key Words: Glottis, imaging technique, larynx, ultrasound, vocal fold.

***Laryngoscope*, 118:1894–1899, 2008**

INTRODUCTION

It is widely accepted that the pliability of the vocal fold cover [epithelium and superficial lamina propria (SLP)] is critical for efficient valving of the glottic air stream in voice production, and that disruption of the normal-layered microstructure is the cause of most dysphonia (hoarseness).[1] Several research groups are pursuing strategies to repair scarred vocal folds by injecting biological and synthetic materials into the damaged layer(s) of the vocal fold.[2] Although this approach seems promising, surgeons have limited means of visualizing, and assessing the location and fate of injected materials during or after surgery. Moreover, the diagnosis, treatment, and outcome evaluation for many other common laryngeal diseases, such as dysplasia, cancer, and papillomatosis would also benefit from improved imaging of the extent of the diseased tissue. Computed tomography and magnetic resonance imaging are often used to supplement microlaryngoscopy as additional imaging tools. However, these imaging technologies cannot resolve the relationship between surface pathology and the underlying layered microstructure. Therefore, there is substantial need for a method that would allow surgeons to analyze the epithelium and lamina propria of the vocal folds preoperatively, intraoperatively, and postoperatively. Burns et al.[3] explored polarization-sensitive optical coherence tomography for vocal fold imaging. Their images showed a clear distinction between the epithelium, the more homogeneous SLP, and the outer surface of the vocal ligament. However, they mentioned that the most significant shortcoming of optical coherence tomography was a depth limitation of about 1.2 mm.

Over the past two decades, high-frequency miniaturized ultrasound transducers have facilitated higher resolution imaging. High-frequency systems typically operate at greater than 30 MHz, and can image living tissue and blood flow with near-microscopic resolution.[4] The cost of the improved resolution is a loss in depth of penetration, and as a result, use of high-frequency systems has been

primarily limited to superficial imaging with applications in ophthalmology, dermatology, catheter-based intravascular imaging, and small animal models.[5–7] In previous ultrasound imaging studies, it has been found that tissue layers abundant in collagen appear as hyperechoic whereas layers abundant in elastin appear hypoechoic,[8,9] suggesting that if layers of differing fiber type composition or pathology (scar) are present in the vocal fold of a species, ultrasound imaging has the potential to differentiate these layers.

Much of the previous work on the ultrasonic imaging of vocal folds has been carried out with probe frequencies less than 20 MHz, which provide sub-optimal image resolution. Two studies[10,11] used radial scanning probes, and were unable to obtain transverse images of the vocal fold. Higher frequency (>30 MHz) ultrasound imaging[12] offers the potential for resolving more detail within the vocal fold, and recently measurements made with a high-resolution 47 MHz ultrasound biomicroscope system were capable of differentiating the lamina propria from the muscle of the human vocal fold.[13] The aim of this article was to obtain high-resolution images of the calf vocal fold ex vivo and to determine if high-frequency ultrasound is capable of visualizing and distinguishing its key features for human diagnostic and surgical applications. To associate image features directly with actual tissue structure and type, correlation to histological sections of the tissue was made to facilitate identification of the various anatomical layers and injected materials.

MATERIALS AND METHODS

Four freshly excised calf vocal folds that had no obvious pathology were imaged. Hemi-larynges were affixed to a clay block using needles to ensure that the vocal fold tissue would remain stable throughout the imaging and freezing portions of the experiment. To simulate a potential repair strategy for the vocal folds, sub-epithelial injections of liposuctioned human fat and a polymer-based hydrogel were made. The hemi-larynges were imaged using a Vevo 770 high-resolution micro-imaging ultrasound system (VisualSonics, Toronto, Canada) at 40 and 55 MHz. This ultrasound system was primarily developed for echocardiography and anatomic assessment of embryonic phenotype of small animals, and enables real-time high-spatial resolution of in vivo structure down to 30 μm.

The vocal folds were imaged in noncontact mode, submerged in physiological saline in both the horizontal plane (parallel to the anterior-posterior axis of the vocal fold and perpendicular to the rostro-caudal axis of the airway), and in the coronal plane, which provides a section plane transverse to the long axis of the vocal fold (Fig. 1). Imaging could have been performed using a thin layer of water-based gel on the face of the ultrasound transducers to make mechanical contact with the vocal folds, but specimen submersion in fluid provided increased positioning accuracy and convenience as images were captured at multiple locations separated by known increments using an X–Y stage and reference needles. Two needles were placed in each of the vocal folds for orientation to the transverse plane and as positional markers for capturing ultrasound images and subsequent histology.

The probe was advanced in 1 mm increments along the length of the vocal fold. Images at least 10 mm wide by 10 mm deep were recorded, encompassing the epithelium, lamina propria, and underlying muscle. Longitudinal images were obtained by rotating the X–Y stage 90°.

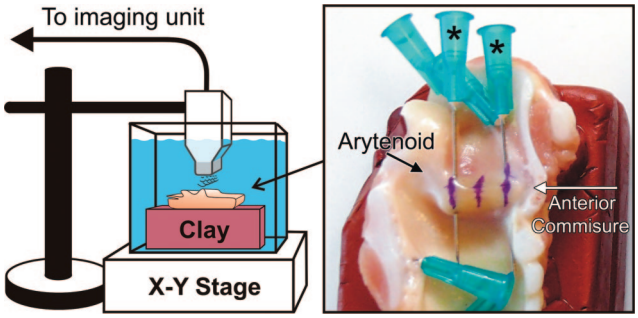

Fig. 1. The experimental setup showing the hemi-larynx fixed to a clay block and immersed in physiological saline for imaging with the ultrasound probe. The left hemi-larynx with needles securing it to a clay block is also shown. Two needles (labeled with asterisks) are inserted from superior to inferior in the transverse plane through the anterior and posterior vocal fold, spaced approximately 10 mm apart.

Histological tissue dye was used to mark the location of particular ultrasound imaging locations for subsequent correspondence with histological sections (see Fig. 1), the larynges were snap-frozen, and the vocal folds were prepared for cryostat sectioning at 12 μm thickness. The sections were stained with H&E, Trichrome and Verhoeff's Van Gieson stains, and images were captured using a microscope at a magnification of 2×. Comparisons between ultrasound images and corresponding histological sections were made to identify anatomical features visible in the ultrasound images, because the layered microstructure of the calf larynx has not been previously described to serve as a point of comparison.

RESULTS

Vocal Fold Structure

Ultrasound images were obtained using 40 and 55 MHz probes in both the transverse and longitudinal planes of section, but only the 40 MHz probe images are presented in this report (unless otherwise specified) because of the greater depth of image resolution achieved at the lower excitation frequency and the greater likelihood that the 40 MHz probe can be miniaturized for application in the confines of the human airway. A comparison of images obtained using the two probes is presented in Figure 2. The 55 MHz transducer provided better resolution images of the vocal fold surface than the 40 MHz transducer, but with the trade-off of degraded image quality deeper into the vocal fold. Using the 55 MHz probe, the epithelial layer and the lamina propria can be visualized as hyperechoic, and can be clearly differentiated from each other by a thin hypoechoic layer. Histological sections showed a thin dense layer of elastin just under the epithelium.

An example of needle location in the transverse plane is shown in an H&E stain histological section (Fig. 3A) and corresponding ultrasound image (Fig. 3B). Another representative transverse section was stained with Trichrome at the mid-musculomembranous level of the calf vocal fold (Fig. 3C). The discernible histology consists of the epithelial surface, the lamina propria, and the muscle. In the corresponding ultrasound image (Fig. 3D), the lamina propria seems as a homogeneous hyperechoic region, and can be distinguished from the less hyperechoic and coarser

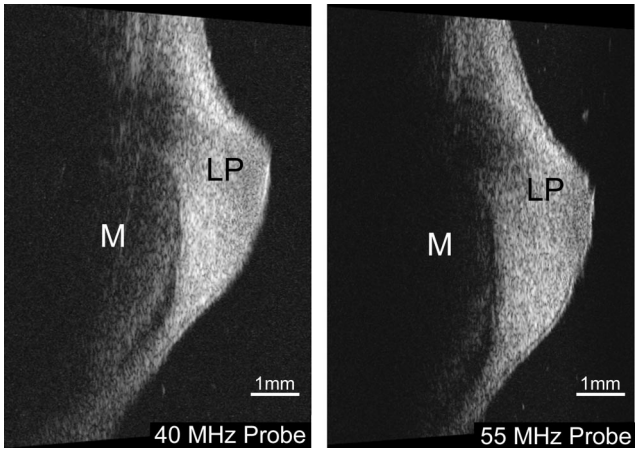

Fig. 2. Transverse vocal fold images from the 40 MHz probe (left) and 55 MHz probe (right) are shown with the medial edge oriented to the right. The lower frequency probe is better able to resolve the vocal fold muscle (M) deep to the lamina propria (LP), but the higher frequency probe provides a more-detailed image of the epithelium.

appearance of the muscle by a narrow hypoechoic region. The epithelial layer was not able to be readily differentiated from the lamina propria in most of the images using the 40 MHz probe. However, on occasions when the epithelial layer was at the focal point of the ultrasound beam, a hypoechoic region could be observed at the edge of the lamina propria that may represent the boundary between the epithelium and lamina propria (Fig. 3B).

The histological sections showed that the lamina propria consisted largely of collagen and elastin fibers where the Trichrome stain highlights collagen as blue and the elastin as red (Fig. 3C). Similarly, a Verhoeff's Van Gieson stain highlighted elastin in the lamina propria (not shown). Appearing at a depth of approximately 2 to 3 mm is a pronounced hypoechoic boundary between the lamina propria and the muscle. Otherwise, the region of lamina propria between the epithelium and the muscle seemed to be homogeneous for the most part, although occasionally some variations in reflectance were observed. However, it was not possible to reliably discern subdivisions of the lamina propria that might correspond to the superficial, intermediate, and deep layers that have been described for the human larynx.

Another H&E stained histological section (Fig. 3E) and the corresponding transverse ultrasonic image (Fig. 3F) are also shown for a level through the posterior vocal fold, both clearly showing the cartilaginous vocal process. Ultrasonically, the vocal process seems as a hypoechoic structure with the lamina propria superficial to it appearing as hyperechoic. Histologically, the lamina propria is clearly distinct from surrounding tissue, but takes a slightly different shape than what is seen in the corresponding ultrasound image, possibly because of freezing artifact and/or slightly oblique ultrasound image capture relative to the histological plane of section.

In the ultrasound images in the transverse plane (Fig. 3B, D, F) there is a noticeable difference between the appearance of the muscle and the lamina propria. The image of the longitudinal section of the vocal fold (Fig. 4) illustrates this more clearly, although no histological sections were prepared for the longitudinal plane. The two needles inserted transversely through the anterior and posterior vocal fold appear in cross-section when the vocal fold was imaged longitudinally, and their artifacts are

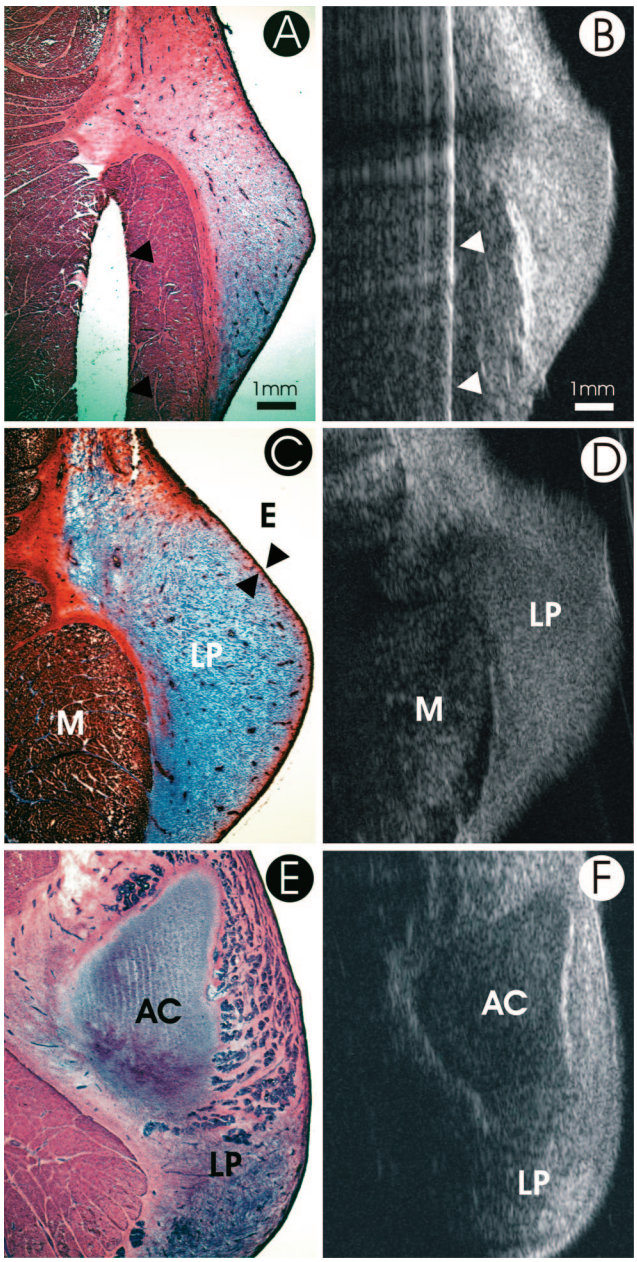

Fig. 3. Transverse vocal fold images are shown with the medial edge oriented to the right. Photomicrographs of stained tissue sections (A, C, and E) with corresponding ultrasound images (B, D, and F) show common anatomical features. Image A shows an H&E stained section with indication (black arrowheads) of where it was cut by a hypodermic needle placed for orientation. Artifacts from the needle in the ultrasonic image (B; white arrowheads) are clearly visible as a white line with successive echoes caused by multiple reverberations. Trichrome stained sections (C, E) with their corresponding ultrasonic image (D, F) show the boundary between lamina propria (LP) and muscle (M) in the transverse plane at the mid-musculomembranous vocal fold (C, D), and show the vocal process of the arytenoid cartilage, appearing as a rounded triangle, (labeled AC) in a section taken more posteriorly. The epithelial layer is labeled "E" in image C.

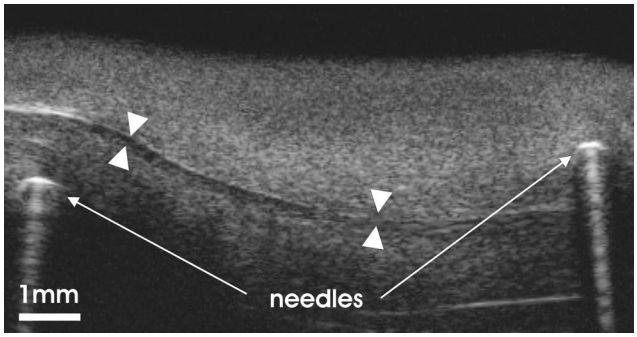

Fig. 4. Visualization of the boundary (indicated by arrows) between the lamina propria and muscle in the longitudinal plane. Greater brightness of boundary above the left needle is because of its normal angle to ultrasound beam. Two reference marker needles, shown in Figure 1, are visible as bright arcs in cross section, followed by echo artifacts deeper into the tissue.

visible at each end on the vocal fold. They are spaced approximately 10 mm apart.

In the images of the longitudinal plane, the thickness of the lamina propria varied from approximately 1 mm at the ends to 3 mm at the center of the vocal folds. The hypoechoic boundary between the lamina propria and the muscle is again clearly visible along most of the length of the vocal fold. This layer, continuous with the perimysium of the muscle, was rich in collagen and elastin fibers, and had a much higher tissue density than the more superficial portion of the lamina propria. However, a region in the far left of the boundary seems as hyperechoic (Fig. 4). This is most likely due to a higher amount of reflectance as the surface is at a right angle to the ultrasound beam when imaged longitudinally. As in the transverse ultrasound images (Fig. 3) the lamina propria also has a more homogeneous and finer appearance than the muscle in the longitudinal plane (Fig. 4). Near the mid-level of the vocal fold, there is a region of the lamina propria that seems to be somewhat less echoic, and this may be due to a different distribution of elastin and collagen fibers.

Imaging Injected Materials

For another vocal fold, liposuctioned human fat was injected beneath the epithelium. The fat is clearly visible in the histological section (Fig. 5A), and it seems as a hyperechoic region in the ultrasound image using the 40 MHz probe (Fig. 5B). An incomplete histological section was obtained from a location in the vocal fold where an injection of hydrogel was made (Fig. 5C). It is evident that the hydrogel did not survive histological processing. However, the epithelium boundary that normally encapsulates the lamina propria is still intact. In the corresponding ultrasonic image (Fig. 5D) the hydrogel injection is clearly visible. It appears as a hypoechoic region with localized hyperechoic regions throughout, likely because of air bubbles. The size of the injection is indicated by the "hole" in the histological section, and from the images it is evident that there is a good correlation between the ultrasound image and the histological section in terms of the size and location of the injection.

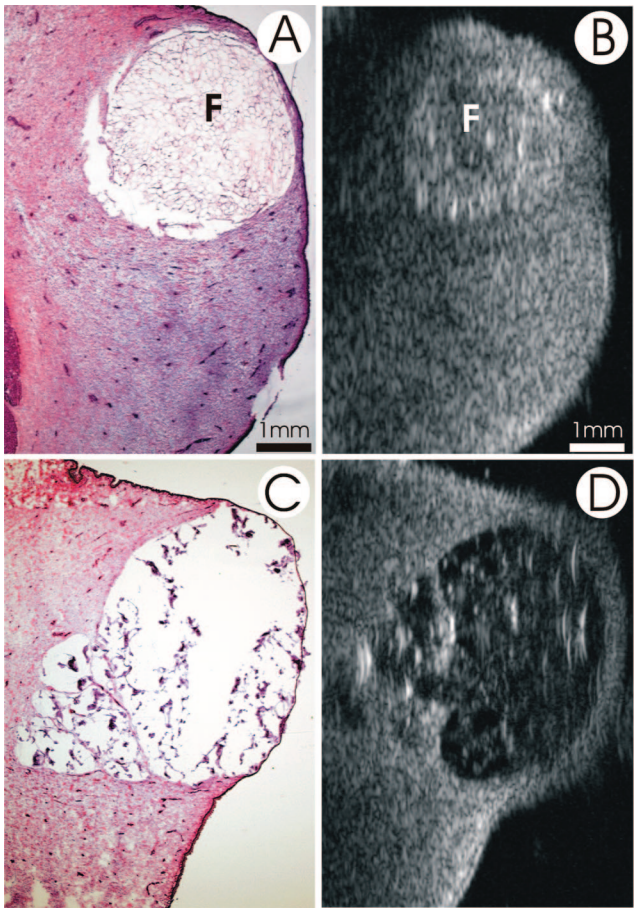

Fig. 5. Visualization of a sub-epithelial fat injection (labeled F) in the lamina propria of the vocal fold in an H&E stained histological section (A) and its corresponding ultrasound image (B). An H&E stained histological section through vocal fold with hydrogel injection (C) shows that the hydrogel did not survive the freezing and cutting process on the cryostat. However, in the corresponding ultrasound image (D) the hydrogel is clearly visible.

DISCUSSION

Our results demonstrate that high-frequency ultrasound is capable of identifying some of the key features of the calf vocal fold. As described in previous reports, the epithelium appeared as a hyperechoic region, likely because of surface reflection off the vocal fold. The lamina propria was separated from the epithelium by a hypoechoic layer that may be attributed to a high concentration of elastin fibers observed just under the epithelium. The muscle appeared as slightly less hyperechoic and coarser than the lamina propria; this may be attributed to the presence of larger tissue structures such as muscle fascicles. A thin hypoechoic boundary separated the muscle and lamina propria along the length of the vocal fold, and histological sections demonstrated that this layer was rich in collagen and elastin fibers. It is thus likely that this layer corresponds to an anatomical structure and may represent muscle epimysium and/or a thin vocal ligament based on its location. The epithelium was more consistently visualized with the 55 MHz probe; however, imaging of deeper layers with this probe was less successful

than with the 40 MHz probe. This reflects the well-known trade-off between resolution and depth of penetration, which is the characteristic of ultrasound imaging. Therefore, 40 MHz may be near the upper limit in frequency for probes that are useful for imaging the full thickness of the vocal fold, whereas higher frequencies could be useful for assessing superficial pathologies such as dysplasia or squamous cell carcinoma with greater resolution. A recent study[13] using a 47 MHz center frequency single ultrasound transducer showed that the lamina propria could be distinguished from the underlying muscle based on acoustic parameters. This is a proof-of-concept that at least frequencies up to 47 MHz may be capable of adequate penetration.

Deep to the epithelium is the lamina propria, which in humans has variations in collagen, elastin, and other extracellular matrix constituents as a function of depth. These variations have led investigators to subdivide the lamina propria into superficial, intermediate, and deep layers.[15] The ultrasound images of the calf vocal fold, however, showed a relatively homogeneous lamina propria extending from the subepithelial hypoechoic zone to the hypoechoic zone seen superficial and adjacent to the muscle layer. We did not observe any distinction between superficial and intermediate layers in our histological sections, which is consistent with the homogeneity of the ultrasound reflectance across most of vocal fold depth. The relatively undifferentiated structure of the calf vocal fold could be caused by immaturity (the human lamina propria lacks the features of the adult layered lamina propria until ~13 years of age[16]), or it might simply reflect anatomical differences between human and bovine species.

The resolution of vocal fold anatomy we obtained with the 40 and 55 MHz probes seems to be an improvement over earlier studies of vocal folds using lower frequency instruments. Arens et al.[10] showed that endolaryngeal high-frequency ultrasound is capable of estimating the infiltration of laryngeal lesions using 10 and 20 MHz probes. However, the image quality was not sufficient to clearly identify the three-dimensional relationship between lesion and underlying vocal fold microstructure. Optimizing resolution is clearly essential if this information is going to be used to guide phonosurgical management of glottic cancer. Currently, unnecessarily broad surgical margins are often taken in the treatment of T1 glottic carcinoma because of unknown lesion depth.[17] A reduction in vocal fold mucosal wave amplitude, or propagation observed on stroboscopy is not a reliable indicator of the presence of cancer or its depth of invasion.[14] Although suboptimal, the current approach comprises microlaryngoscopic visualization of the glottal surfaces coupled with palpation and saline infusion.[18] However, this approach requires an experienced surgeon and is not easily quantified or mapped. Thus, an imaging technique that provides accurate lesion depth information would enable accurate diagnosis, greatly facilitate the maintenance of ultra-narrow surgical margins, and also enable the outcome to be easily evaluated. It seems that the increase in frequency to 40 or 55 MHz may provide the extra resolution needed, but this needs to be tested with human specimens. In another previous study, Tamura et al.[11] showed some promising results, applying a radial scanning 20 MHz miniaturized probe to 10 human cadaver larynges and reported that they could distinguish three layers within the lamina propria. Their study was limited, however, by use of a radial probe that requires interpolation of radial image lines, resulting in lower resolution images. Another drawback to rotating systems is that friction (e.g., due to cannula bending) can cause variations in the rotation rate, which results in image distortion.

As mentioned earlier, current strategies for repairing damaged vocal folds include the injection of biological and synthetic materials into the SLP. In this work, we demonstrate that high-frequency ultrasound is capable of identifying injections of fat (hyperechoic) and a hydrogel (hypoechoic), and that these materials showed up in good contrast to the contiguous tissues. Therefore, a high-frequency ultrasound endolaryngeal probe could allow surgeons to better determine the volume and location of injected substances, as long as they have adequate acoustic contrast.

There are a number of well-known features of ultrasonic imaging that make it well suited to diagnostic imaging of vocal folds. The clinician can interactively examine the tissue noninvasively and in real time. Dynamic information as the probe is moved around and the tissues are deformed may provide additional information as it does when examining other tissues with ultrasound. Real-time adjustments to optimize image contrast, resolution, and field of view are also possible. The main challenge to clinical implementation of high-frequency ultrasound to vocal fold imaging is probe miniaturization. Work is ongoing in a number of laboratories to develop miniaturized high-frequency transducers.[11,13] Cannata et al.[19] are developing a small 35 MHz piezo-composite ultrasound array for medical imaging, and so far have demonstrated resolution exceeding 50 μm axially and 100 μm laterally. Recently there is also the Food and Drug Administration approved 45 MHz intravascular ultrasound Revolution from Volcano. These smaller high-frequency transducers indicate that useful endolaryngeal high-frequency ultrasound imaging is becoming a possibility for the first time.

In this report, we have undertaken an ex vivo, proof-of-concept evaluation of high-frequency ultrasound imaging of bovine vocal folds using equipment that is currently too large for endolaryngeal placement in human subjects. We believe that the images obtained show what may be obtained in vivo with new and upcoming miniaturized ultrasound technology. This is the first time that the subsurface features of the vocal fold have been imaged with such clarity using ultrasound. Based on this study, it is our belief that miniaturization of this technology will eventually provide surgeons with a view of the microstructure of the human vocal fold of sufficient quality to aid in diagnosis, surgical procedures, and outcome analysis.

Acknowledgments

The authors thank Gerardo Lopez-Guerra, James Burns, Susan Erdman, and VisualSonics for their assistance with tissue preparation and ultrasound imaging.

BIBLIOGRAPHY

1. Zeitels SM, Healy GB. Laryngology and phonosurgery. *N Engl J Med* 2003;349:882–892.

2. Zeitels SM, Blitzer A, Hillman RE, Anderson R. Foresight in laryngology and laryngeal surgery: a 2020 vision. *Ann Otol Rhinol Laryngol Suppl* 2007;198:2–16.
3. Burns JA, Zeitels SM, Anderson RR, Kobler JB, Pierce MC, de Boer JF. Imaging the mucosa of the human vocal fold with optical coherence tomography. *Ann Otol Rhinol Laryngol* 2005;114:671–676.
4. Che'rin E, Williams R, Needles A, et al. Ultrahigh frame rate retrospective ultrasound microimaging and blood flow visualization in mice in vivo. *Ultrasound Med Biol* 2006;32: 683–691.
5. Lockwood GR, Turnbull DH, Christopher DA, Foster FS. Beyond 30 MHz: applications of high-frequency ultrasound imaging. *IEEE Eng Med Biol* 1996;15:60–71.
6. Foster FS, Zhang MY, Zhou YQ, et al. A new ultrasound instrument for in vivo microimaging of mice. *Ultrasound Med Biol* 2002;28:1165–1172.
7. Konig A, Oepke M, Leibig M, Klauss V. Coronary plaque classification using intravascular ultrasound. *Clin Res Cardiol* 2007;96:514–518.
8. Kobayashi T. Ultrasonic tissue characterization. *Medicina (B Aires)* 1981;18:1132–1135.
9. Yokoi H, Honda N, Nakama S, et al. The tissue characterization by ultrasonography in relation to the content of collagen fibers of gastric wall, skin and muscle. *J Nissei Hosp* 1984;12:13–17.
10. Arens C, Glanz H. Endoscopic high-frequency ultrasound of the larynx. *Eur Arch Otol Rhinol Laryngol* 1999;256:316–322.
11. Tamura E, Kitahara S, Kohno N. Intralaryngeal application of a miniaturized ultrasonic probe. *Acta Otol Laryngol* 2002;122:92–95.
12. Szabo TL. *Diagnostic Ultrasound Imaging: Inside Out.* Burlington, MA: Elsevier Academic Press; 2004:71–95, 213–295.
13. Huang C-C, Sun L, Dailey SH, Wang S-H, Shung, KK. High-frequency ultrasonic characterization of human vocal fold tissue. *J Acoust Soc Am* 2007;122:1827–1832.
14. Gray SD, Hirano M, Sato K. Molecular and cellular structure of vocal fold tissue. In: Titze IR, ed. *Vocal Fold Physiology*. San Diego, CA: Singular Publishing Group; 1993:1–35.
15. Hartnick CJ, Rehbar R, Prasad V. Development and maturation of the pediatric human vocal fold lamina propria. *Laryngoscope* 2005;115:4–15.
16. Zeitels SM, Hillman RE, Franco RA, Bunting GW. Voice and treatment outcome from phonosurgical management of early glottic cancer. *Ann Otol Rhinol Laryngol Suppl* 2002;190:3–20.
17. Colden D, Zeitels SM, Hillman RE, Jarboe J, Bunting G, Spanou K. Stroboscopic assessment of vocal fold keratosis and glottic cancer. *Ann Otol Rhinol Laryngol* 2001;110:293–298.
18. Zeitels SM. Premalignant epithelium and microinvasive cancer of the vocal fold: The evolution of phonomicrosurgical management. *Laryngoscope* 1995;105(suppl 67):1–51.
19. Cannata JM, Wlliams JA, Zhou Q, Ritter TA, Shung KK. Development of a 35-MHz piezo-composite ultrasound array for medical imaging. *IEEE Trans Ultrason Ferroelectr Freq Control* 2006;53:224–236.