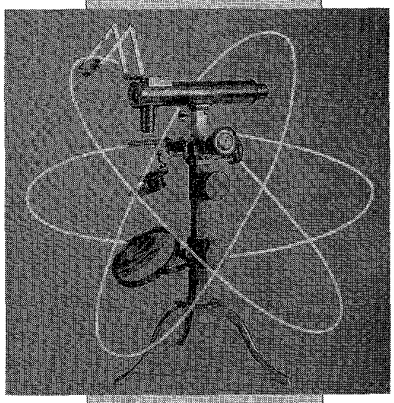

Characterizing Biological Tissue Using Scanning Laser Acoustic Microscopy

SLAM technique offers high resolution for determining the properties of individual layers

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Scanning laser acoustic microscopy (SLAM) uses the principles of plane wave sound propagation for imaging and quantification of acoustic properties[1]. Briefly, the principle of operation is that sound waves are generated with a piezoelectric transducer and transmitted to an object. The sound waves traverse the object, in accordance with its acoustic properties, and then strike an optical mirror-like coverslip that serves as an acoustic detector. Simultaneously, a laser beam is scanned across the coverslip to produce an image. The SLAM can be operated in three modes: image, acoustic and interference. With the first, images comparable to those of optical microscopy can be obtained; with the second, images containing information of the acoustic attenuation can be obtained; and with the third mode, images are obtained from which the speed of sound can be calculated[2]. The resolution of SLAM depends on the sound wave frequency and the laser spot size, and resolutions better than 20 μm can be achieved[3]. Conventional tissue fixation and staining are not needed for SLAM imaging, and thus, living cells and tissue can be studied with this technique.

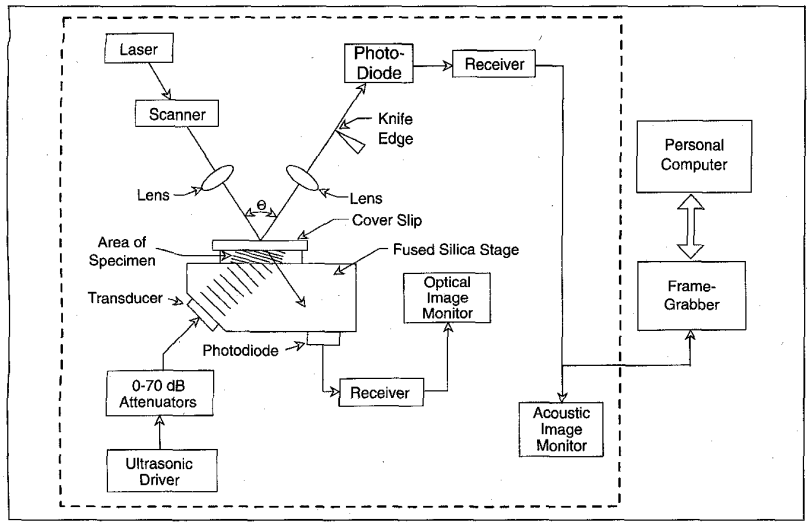
The SLAM technique has found extensive use in assessment of acoustic properties of tissues such as skin[4], kidney[5] and liver[6], and is a candidate for use in the field of tissue characterization at the microscopic level. Tissue characterization can be defined as the identification of physical properties of a tissue that reflect its type so well that it may be identified on those alone. No single parameter of tissue properties can possibly be used to identify a type of tissue, but the ability of the SLAM to produce simultaneously optical images and images from which the acoustic properties of the specimen can be calculated, facilitate its use in this field of biology.

To introduce the SLAM technique as a method for measuring the speed of sound in tubular layered organs, and possibly distinguish between the layers by this measure, we chose the guinea-pig urethra, due to its relatively simple layered structure. To obtain more data of the acoustic properties of tissue, the attenuation coefficient was determined for the wall in toto.

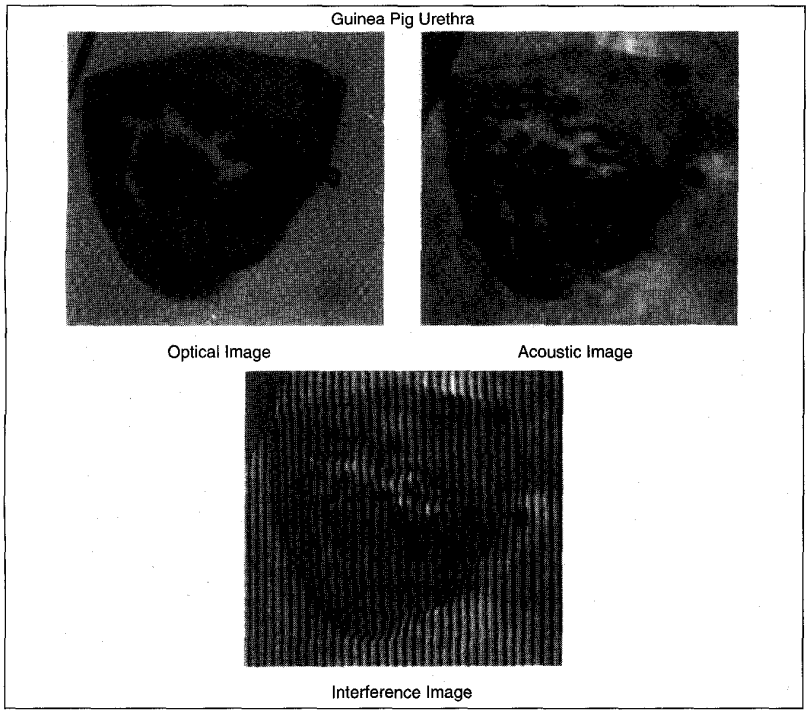
Material and methods

Specimen preparation

An 800 g female guinea-pig was euthanized by cervical dislocation and the abdomen was opened by a long midline incision. The urethra was located and a segment was excised beginning 2 mm from its junction with the bladder to 3 mm from its external orifice. The segment was placed in a cryotube holding a 0.9% NaCl solution. The tube was snap-frozen in liquid nitrogen and stored at -70°C until the time of investigation. Prior to study, the segment was thawed and trimmed, and cut in a cryo-microtome. Four transverse specimens, 60, 80, 100, and 120 μm thick for measurements of the acoustic attenuation and six transverse 100 μm thick specimens for measurements of the speed of sound were made. Each specimen was placed on the SLAM stage and allowed further equilibration to room temperature for 3 min before measurements were made. The 0.9% NaCl solution was also used as a coupling medium between the transducer and the specimen, and served as a reference medium for calculation of acoustic properties (see below).



1. Block diagram of the SLAM setup.



2 Photographs of a guinea-pig urethra taken with the SLAM technique at 100 MHz; (a) optical image, (b) acoustic image, (c) interference image.

Scanning laser acoustic microscopy

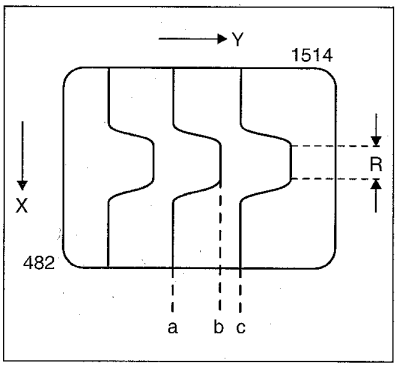
The microscope. The technical details and operating principles of the SLAM used in this study (Sonomicroscope, Sonoscan, Inc., Bensenville, Illinois) have previously been described in detail[2]. The three SLAM modes produce three different images. In the optical mode, a focused laser beam scans the specimen, which is placed on the microscope stage with its upside covered with a mirror-like slip (Fig. 1). The beam is transmitted through the slip and specimen, and its intensity is detected by a photodiode. The signal is electronically processed and transmitted to a TV-monitor to give an image comparable to that of conventional optical microscopy (Fig. 2A). In the acoustic mode, the specimen is insonified with an incident ultrasonic plane wave generated by a 100 MHz piezo-electric sound transducer located below the specimen (Fig. 1). The sound waves traverse the specimen and strikes the surface of the coverslip. The sound field holds information of the acoustic properties of the specimen and, in accordance with those properties, causes the slip to emit a dynamic ripple pattern of angular periodic deflections. The deflections are detected by the scanned laser beam which is reflected to a photodetector and processed into an image shown on the TV-monitor (Fig. 2b). From this image, the attenuation of the sound waves having traversed the specimen is calculated. In the interference mode, the laser beam ripple pattern is detected by the same photodiode as in the acoustic mode and is then mixed with a 100 MHz reference signal (Fig. 1) to produce an interference image, which is also shown on the TV-monitor (Fig. 2C). From this image, the speed of sound is calculated.

Calculating acoustic properties. The attenuation coefficient, the attenuation of sound energy per unit distance of travel, is estimated from calculations of the loss of sound wave energy in the specimen [7]. In principle, the specimen signal amplitude is compared to that of the coupling medium. To approach uniformity, the acoustic image is subdivided into 64 areas of approximately 400 by 250 μm (each 96 by 32 pixels) for which the signal voltage amplitudes (V) are averaged[7]. A minimum of five V values are recorded at different locations in both the reference medium and the specimen to calculate a minimum of five insertion loss (IL) values:

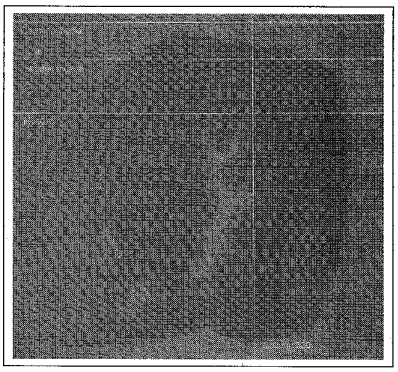
$$IL = V_s - \langle V_r \rangle \quad (dB) \tag{1}$$

where V_s and V_r are the signal voltage amplitudes of the specimen and reference medium, respectively. To calculate the attenuation coefficient, which is equal to the slope of the IL-thickness curve[7], measurements were done on 60, 80, 100 and 120 μm thick specimens.

The speed of sound is calculated from the shift of the lines in the interference image, where the lines shift to the right when the sound waves enter an object having a higher speed of sound relative to the coupling medium (Fig. 3). Quantitative speed of sound profiles are created from analysis of several image regions in different loci. The speed of sound in the specimen (c_s) is calculated in relation to that of the coupling medium (c_{cm}) which



3. Schematic representation of the interference shift N = ab/ac



4. The speed of sound measured in the outer muscular coat and the inner mucosa along the vertical line.

is 1522 m s^{-1}. This is done by use of the spatial frequency domain technique (SFDT) [10]:

$$c_s = \left(\frac{c_{cm}}{\sin\theta_{cm}}\right)\sin\left(\frac{1}{\tan^{-1}\left(\frac{1}{\tan\theta_{cm}} - \frac{N\lambda_o}{T\sin\theta_{cm}}\right)}\right) (m\,s^{-1}) \quad (2)$$

where θ_{cm} is the angle between the direction of propagation of sound in the coupling medium and the normal, λ_{cm} is the wavelength of sound in the coupling medium, and T is the specimen thickness. N is the shift of the interference lines (Fig. 3). Measurements of the speed of sound were done in one location in each layer of the wall (Fig. 4). The attenuation coefficient was estimated by use of linear regression of the *IL*-thickness data. The speed of sound values for the individual layers were compared by means of the Mann Whitney test. The values given are the means and their standard deviation (SD).

Results

The images obtained are shown in Fig. 2. The optical image shows a typical cross-section of the urethra with a central lumen, a relatively thin rim of mucosa, and a thicker muscular coat. (Fig. 2a). In the acoustic image, the mucosa has a somewhat darker contrast than the muscle coat, suggesting that the former has a higher attenuation than the latter (Fig. 2b). In the interference image, the interference lines shift to the right, entering the muscular coat from the coupling medium, and a small shift to the left may be seen when it enters the mucosa (Fig. 2c). This relationship suggests that the speed of sound is higher in the muscle coat than in the mucosa, which is also the case: In the former speed is 1635 ± 4 and in the latter 1580 ± 6 m s^{-1} ($p<0.05$). The attenuation coefficient for the wall in toto is 112 dB mm^{-1} ($r = 0.94$).

Discussion

Our results show that with SLAM it is possible to image sections of the guinea-pig urethra and distinguish its layered topography. Most importantly, the urethral wall can be quantitatively characterized and its layers distinguished by use of the SLAM speed of sound profile. Comparable quantitative data for the urethra are sparse, but values of the speed of sound similar to the ones found for the muscle coat here have been described previously [9]. A possible explanation for the higher speed of sound in the muscular coat compared to the mucosa may be that the former contains relatively more connective tissue fibers which have been shown to have a relatively high speed of sound. The attenuation coefficient highly depends on the sound frequency at which it is measured [7]. A previous study found attenuation coefficients in the range of 30-60 dB mm^{-1} in canine skin at 100 MHz, and also demonstrated some correlation between the amount of collagen fibers and the attenuation coefficient [9]. The difference between those values and the one found here may thus partially be explained by differences in the amount of collagen and possibly by other differences of tissue constituents. The accuracy and error of SLAM for speed measurements have previously been estimated to ±2.9 and ±0.4 % (worst case), respectively (9), These results further indicate that the difference in the speed of sound between the muscular coat and mucosa can not be explained by measurement uncertainties. We conclude that the SLAM technique may contribute to tissue characterization, providing high resolution measurements of tissue acoustic properties. Future SLAM studies should include quantitative investigations of the relation between tissue constituents and acoustic properties.

Acknowledgments

The authors acknowledge financial assistance from NIH grants CA09067 and HD21867 and from the University of Illinois Value-added Research Opportunities Program and Karen Elise Jensens Fond, Copenhagen, Denmark. The technical assistance from Dr. Amjad A. Safvi, Ms. Jia Yao and Professor James F. Zachary at the University of Illinois is also acknowledged. Jens Kristian Poulsen, M.Sc., SAM Laboratory, Institute of Experimental Clinical Research, University of Aarhus, is thanked for a critical review.

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