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Ultrasound elastic tensor imaging: comparison with MR diffusion tensor imaging in the myocardium

Wei-Ning Lee\(^1\)\(^2\)\(^3\), Benoît Larrat\(^1\)\(^2\)\(^3\), Mathieu Pernot\(^1\)\(^2\)\(^3\)\(^4\) and Mickaël Tanter\(^1\)\(^2\)\(^3\)

\(^1\) Institut Langevin, ESPCI ParisTech, Paris, France
\(^2\) CNRS, UMR 7587, Paris, France
\(^3\) INSERM, U979, Paris, France

E-mail: mathieu.pernot@espci.fr

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Abstract

We have previously proven the feasibility of ultrasound-based shear wave imaging (SWI) to non-invasively characterize myocardial fiber orientation in both in vitro porcine and in vivo ovine hearts. The SWI-estimated results were in good correlation with histology. In this study, we proposed a new and robust fiber angle estimation method through a tensor-based approach for SWI, coined together as elastic tensor imaging (ETI), and compared it with magnetic resonance diffusion tensor imaging (DTI), a current gold standard and extensively reported non-invasive imaging technique for mapping fiber architecture. Fresh porcine (\(n = 5\)) and ovine (\(n = 5\)) myocardial samples (20 × 20 × 30 mm\(^3\)) were studied. ETI was firstly performed to generate shear waves and to acquire the wave events at ultrafast frame rate (8000 fps). A 2.8 MHz phased array probe (pitch = 0.28 mm), connected to a prototype ultrasound scanner, was mounted on a customized MRI-compatible rotation device, which allowed both the rotation of the probe from −90° to 90° at 5° increments and co-registration between two imaging modalities. Transmural shear wave speed at all propagation directions realized was firstly estimated. The fiber angles were determined from the shear wave speed map using the least-squares method and eigen decomposition. The test myocardial sample together with the rotation device was then placed inside a 7T MRI scanner. Diffusion was encoded in six directions. A total of 270 diffusion-weighted images (\(b = 1000 \text{ s mm}^{-2}\), FOV = 30 mm, matrix size = 60 × 64, TR = 6 s, TE = 19 ms, 24 averages) and 45 \(B_0\) images were acquired in 14 h 30 min. The fiber structure was analyzed by the fiber-tracking module in software, MedINRIA. The fiber orientation in the overlapped myocardial region which both ETI and DTI accessed was therefore compared, thanks to the co-registered imaging system. Results from all ten samples showed good correlation.
The average ETI-estimated fractional anisotropy (FA) values decreased from subendocardium to subepicardium ($p < 0.05$, unpaired, one-tailed $t$-test, $N = 10$) by 33%, whereas the corresponding DTI-estimated FA values presented a change of $-10\%$ ($p > 0.05$, unpaired, one-tailed $t$-test, $N = 10$). In conclusion, we have demonstrated that the fiber orientation estimated by ETI, which assesses the shear wave speed (and thus the stiffness), was comparable to that measured by DTI, which evaluates the preferred direction of water diffusion, and have validated this concept within the myocardium. Moreover, ETI was shown capable of mapping the transmural fiber angles with as few as seven shear wave propagation directions.

(Some figures may appear in colour only in the online journal)

1. Introduction

Imaging the microscopic and macroscopic structure of biological tissues with ultrasound remains a challenge. The fibrous structure of soft tissues, such as the brain and the myocardium, has not been correctly characterized yet by ultrasound techniques. The fiber organization at cellular and higher levels especially plays a major role in the organ function and is therefore important to image for diagnostic purposes.

Myocardium, for example, is primarily composed of myocytes which are arranged into muscle fibers (Humphrey 2002). Quantitative examinations have verified that the myocardial fibers are organized in layers, dominantly lie within the epicardial-tangent plane, and vary their orientation across the heart wall (Streeter and Bassett 1966, Streeter et al. 1969). Viewed from the anterior side of the left ventricle, myocardial fibers are oriented diagonally (upper left–bottom right) near the epicardium, gradually alter their directions counterclockwise to approximately align with the circumferential axis at the midwall and then become oblique (upper right–bottom left) near the endocardium (Streeter and Bassett 1966, Streeter et al. 1969). Such transmural variation of myocardial fiber orientation has relevance to electromechanics of the myocardium (Arts et al. 2001, Hooks et al. 2007, Waldman et al. 1988). Moreover, disarrangement of myocardial fibers has demonstrated its presence in certain cardiac pathologies, for instance, hypertrophy and post-infarction remodeling. Structural changes of the heart may further influence the functional behavior of the myocardium.

Elastic parameters of tissues, such as the shear modulus (or, the stiffness of soft tissues), have been shown to be highly anisotropic in fibrous structures, including skeletal muscles and the myocardium, within which fiber stiffness may be two to three times higher than cross-fiber stiffness (Costa et al. 2001). This stiffness anisotropy stemming from the local fiber architecture is the pivotal basis of the study. In this perspective, non-invasive imaging techniques that are capable of assessing stiffness anisotropy become valuable to aid the quantification and visualization of the myocardial fiber architecture.

Recent advances in wave-based ultrasound imaging have enabled qualitative and/or quantitative mapping of myocardial stiffness. The traveling waves, namely the propagation of local particle motion, inside the myocardium can be induced by an internal source or an external force. Kanai (2005) proposed a technique to capture and analyze an intrinsic pulsive wave that was generated by the closure of the aortic valve at end systole. Through phase analysis, this vibration wave was found to propagate in the interventricular septum from base
to apex in the parasternal long-axis view in a healthy volunteer (Kanai 2005). Phase velocity of the vibration wave was estimated from the dispersion curves, and myocardial viscoelasticity was obtained using the Voigt model in the anti-symmetric Lamb wave mode (Kanai 2005). Shear waves propagating inside the myocardium may also be generated externally. In shear wave dispersion ultrasound vibrometry (Pislaru et al. 2009), a mechanical actuator that vibrated the epicardial surface in open-chest pig hearts in vivo was used to generate harmonic waves, which were tracked with an ultrasound linear array. Dispersion curves of the shear waves were fitted by an anti-symmetric Lamb wave model to yield myocardial viscoelasticity (Pislaru et al. 2009). An alternative way is to remotely induce shear waves in the myocardium by means of radiation force of a focused ultrasound beam (Bouchard et al. 2009, Couade et al. 2011, Nenadic et al. 2011b, Pernot et al. 2011). Nenadic et al. (2011b) used a push transducer to transmit amplitude-modulated focused ultrasound beam and then tracked the resulting shear waves with a pulse-echo transducer to study the viscoelasticity of excised porcine hearts. Bouchard et al. (2009) employed acoustic radiation force impulse (ARFI) excitation beams to generate shear waves on in vivo open-chest canine hearts. In their study, shear wave generation was repeated four times, in each of which shear wave response at a different lateral location was acquired, and shear wave amplitude was estimated (Bouchard et al. 2009). The wave amplitudes at all four lateral positions were then grouped to track the wave propagation (Bouchard et al. 2009). Whereas the two wave-based ultrasound techniques assessed the shear modulus at a given myocardial depth and a certain lateral extent (Bouchard et al. 2009, Nenadic et al. 2011b), shear wave imaging (SWI) developed by our group utilized a single conventional ultrasound array probe to generate intense plane shear waves that propagated in larger axial and lateral range and to image them in full field of view simultaneously in beating hearts in vivo (Couade et al. 2011, Pernot et al. 2011). Full maps of shear wave speed and thus shear modulus of the myocardium could be visualized (Couade et al. 2011, Pernot et al. 2011).

Substantial effort has been made to evaluate myocardial stiffness based on ultrasound shear wave techniques, but whether stiffness anisotropy could be faithfully derived beyond stiffness measurements requires further investigations. An early study has shown the feasibility of an ARFI-based shear wave technique for stiffness anisotropy in a computational simulation scheme and a homogeneous isotropic phantom (Hsu et al. 2003). A later study from our group has demonstrated that SWI enables the quantification of stiffness anisotropy in skeletal muscle in vivo (Gennisson et al. 2010). More recently, SWI has been performed to probe myocardial stiffness anisotropy (i.e. fiber and cross-fiber stiffness at midwall) (Couade et al. 2011), which has inspired the use of SWI for mapping myocardial fiber orientation both in vitro and in vivo (Lee et al. 2010, 2012).

Our previous work (Lee et al. 2012) has shown that SWI could be served as a potential non-invasive technique for estimating and visualizing transmural fiber directions and that the SWI-estimated fiber angles of the excised porcine myocardium were well correlated with those measured by histology ($r^2 = 0.91 \pm 0.02$, $p < 0.0001$). The labor-intensive histological procedure permitted us to examine up to 25 radial locations across the myocardial wall; in contrast, SWI could measure fiber angles at more than 200 radial sites. Evidently, histology was not a practical tool to assess the spatial resolution of SWI in fiber angle estimation. Therefore, in this study, we resort to employ magnetic resonance imaging (MRI) to validate the proposed ultrasound shear-wave-based technique. Currently, there exist two major MRI techniques for the study of tissue anisotropy: magnetic resonance elastography (MRE) in the breast (Sinkus et al. 2005), human biceps (Papazoglou et al. 2006) and brain (Larrat et al. 2007), and diffusion tensor imaging (DTI). The former technique has also been performed to assess the shear stiffness in the myocardium (Kolipaka et al. 2010). The latter is currently the gold standard for non-invasive mapping of tissue structures in deep organs. DTI quantifies
the diffusion anisotropy of water molecules in tissues and is one of the various MR diffusion techniques which has been extensively investigated for its capability in mapping 2D and even 3D myocardial fiber architecture (Hsu et al 1998, Reese et al 1995, Scollan et al 1998, Tseng et al 2006, Wu et al 2006) and provides higher spatial resolution than histology with less manual and destructive procedure.

Similar to DTI, where higher diffusion rate occurs as water molecules diffuse along the fibers, SWI relies on the physical phenomenon that shear waves propagate faster along the fibers than in the transverse directions to examine fiber orientation. The shear wave speed is associated with its propagation direction with respect to the local fiber direction as well as the local stiffness. As demonstrated in our previous study, SWI estimates myocardial fiber orientation based on the speed anisotropy of shear waves propagating inside the myocardium (Lee et al 2012). At each depth of interest, maximum shear wave speed was sought among all the propagation angles performed. Such maximum-based strategy together with limited angular step resolution of the shear wave propagation resulted in low fiber angle precision and was susceptible to false peaks. To overcome the aforementioned issues, we hereby propose a new approach which solves partially the elastic wave equation in the tensor form through the least-squares method and outputs directly both the elasticity tensor and the high-precision fiber angle estimates. This elastic-tensor-based approach replaces the maximum-based strategy and is integrated with the core of the standard SWI technique, including shear wave generation and analysis, to constitute a generalized ultrasound imaging technique, elastic tensor imaging (ETI), for the assessment of the fiber structure in anisotropic soft tissues.

The goal of the paper is hence to establish ETI for the examination of anisotropic tissues with better accuracy and to validate it experimentally on myocardial samples as its initial attempt. The theoretical framework of ETI for the simultaneous estimation of the local elasticity tensor and fiber angles is first developed and followed by the experimental method. Results are obtained from in vitro myocardium and compared to DTI in terms of fiber directions and fractional anisotropy (FA). Finally, the robustness of the ETI technique with the number of propagation directions required for the retrieval of fiber angles is investigated.

2. Methods

2.1. Basic shear wave properties

The core of the ETI technique is the employment of the shear wave, which is defined as a mechanical wave whose polarization (i.e. particle displacement direction) is perpendicular to its propagation direction. Shear waves propagate at a speed which is linked to both the propagation direction and the material properties of the medium (Royer and Dieulesaint 2000). In soft tissue, shear wave speed ranges between 1 and 10 m s−1. In anisotropic media, such as myocardium, the shear wave propagation speed (v) is a function of the angle (θ) between the shear wave propagation and the muscle fiber directions (figure 1) (Royer and Dieulesaint 2000). Presented in our previous study and illustrated here in figure 1(b), maximum and minimum speeds occur when the shear wave propagates along (θ = 0°) and across (θ = ±90°) the fibers, respectively. Such shear wave speed anisotropy is the fundamental of this study. In order to have a full spectrum of the shear wave propagation speed, a series of shear waves with different propagation directions are generated (section 2.3).
Figure 1. An illustration of the shear wave propagation in a medium with preferential fiber arrangement ($x_{rr}$-axis): (a) a shear wave which is polarized ($u$) in parallel to the $x_{rr}$-axis and propagates in the $x_{rr}$-$x_{cc}$ plane at $\theta$ angle between the propagation direction ($n$) and the $x_{rr}$-axis; (b) the theoretical group velocity ($v$) of the shear wave shown in (a) as a function of $\theta$. $x_{rr}$, $x_{cc}$, and are the fiber, cross-fiber and radial directions in the local fiber coordinates.

2.2. Experimental setup

A system of local cardiac coordinates ($x_c$, $x_l$, $x_r$) was defined, with the circumferential ($x_c$), longitudinal ($x_l$) and radial ($x_r$) axes mutually perpendicular. The longitudinal axis ($x_l$) was firstly determined based on the axis that passed through the aortic root and the apex. The circumferential axis ($x_c$) was orthogonal to the longitudinal axis and tangential to the epicardial surface, and the radial axis ($x_r$) was the normal of the epicardial plane (figure 2(a)). Following the convention employed in the literature (Streeter and Bassett 1966, Streeter et al 1969), the myocardial fiber angles ($\alpha$) were defined between $-90^{\circ}$ and $90^{\circ}$ with $0^{\circ}$ denoting the fibers that aligned with the circumferential axis ($x_c$) as shown in figure 2(a). Viewed from the anterior side of the left ventricle, fibers that rotated clockwise (or counterclockwise) from the circumferential ($x_c$) to the longitudinal axes ($x_l$) of the local cardiac coordinates were assigned negative (or positive) angles (figure 2(a)). This denotation was adapted throughout our study. Based on this angle definition, it has been known that midwall fibers were generally oriented along the circumferential axis, whereas subepicardial and subendocardial fibers were found to respectively exhibit negative and positive angles (figure 2(a)).

2.2.1. In vitro porcine myocardium preparation. Ten fresh myocardial samples of $20 \times 20 \times 30$ mm$^3$ were dissected from the excised porcine ($N = 5$) and ovine ($N = 5$) hearts. A cross-marker whose orientation matched the local cardiac circumferential-longitudinal coordinates was labeled on each myocardial sample. Each specimen was fully embedded inside a cylindrical mold (radius: 13.5 mm; height: 60 mm) filled with a 25 $^\circ$C degassed 8% gelatin solution. The entire mold was then placed in a 4 $^\circ$C refrigerator for the gelatin solution to congeal. The epicardium of the myocardial sample was situated 30 mm below the surface of the gelatin phantom (figure 2(b)).

2.2.2. In vivo ovine heart model. An in vivo examination of the myocardial fiber orientation using ETI was also performed on the mid-anterior region of a beating ovine heart. The in vivo
Figure 2. Coordinates and imaging system setup: (a) a schematic of myocardial fiber orientation, cardiac coordinates ($x_l$, $x_c$, $x_r$) and an ultrasound probe; $\alpha$ and $\beta$ denote the myocardial fiber orientation and the probe rotation angle, respectively. The local cardiac coordinates were defined with the longitudinal axis ($x_l(\alpha, \beta = \pm 90^\circ)$ determined based on the axis that passed through the aortic root and the apex. The circumferential axis ($x_c(\alpha, \beta = 0)$) was orthogonal to the longitudinal axis and tangential to the epicardial surface. (b) A myocardial sample of $20 \times 20 \times 30$ mm$^3$ embedded in a gelatin phantom (radius: 13.5 mm; height: 60 mm) and an MR animal specimen holder, integrated with an MR-compatible rotation device and an ultrasound probe for inter-modality co-registration.

The ovine cardiac experimental procedure was approved prior to use by the Institutional Animal Care and Use Committee of l’Hôpital Européen Georges Pompidou (PARCC) according to the European Commission guiding principles (2010/63/EU). At the termination of this in vivo experiment, the ovine heart was excised, and the mid-anterior wall region was examined by DTI and re-assessed by ETI in an in vitro configuration.

2.2.3. Magnetic resonance imaging (MRI) compatible rotation device. A rotation device (figure 2(b)) onto which the ultrasound probe was mounted was required for the realization of different propagation directions of the shear waves. To ensure the secured position of the myocardial sample and the co-registration between imaging modalities, this rotation device along with the animal specimen holder (figure 2(b)) was designed to be compatible with the high field MRI scanner used (section 2.4.1). This manual rotation device allowed the rotation of the ultrasound probe from $-90^\circ$ to $90^\circ$ in $5^\circ$ increments. The ultrasound probe coordinate system coincided with the local cardiac coordinates ($x_l$, $x_c$, $x_r$) (section 2.2). The aperture was aligned with $x_l$ of the myocardial sample at the probe angle ($\beta$) of $\pm 90^\circ$ and with $x_c$ at $\beta$ of $0^\circ$ (figure 2(a)).

2.3. Elastic tensor imaging (ETI)

2.3.1. Principles. As introduced in section 2.1, the utilization of the shear wave is the key to deriving myocardial fiber orientation. Based on the SWI technique exploited in our previous study on myocardial anisotropy (Lee et al 2012), ETI is comprised of three main elements: (1) shear wave generation by acoustic radiation force, (2) shear wave speed estimation and (3) fiber angle retrieval. The major novelty of ETI lies in the employment of a tensor-based method (explained in detail in section 2.3.4) to improve the robustness and precision of the
fiber angle estimation. In order to differentiate this new approach from the previous maximum strategy used, we have thereby coined the new methodology as ETI. This also reflects the fact that ETI achieves the simultaneous estimation of both local elastic constants and fiber angles through a tensor-based approach.

ETI is essentially a technique that involves one single conventional ultrasound probe to both generate and image the shear waves. Focusing an ultrasound beam at a target spot inside the tissue creates acoustic radiation force, which induces shear waves that are polarized in parallel with but propagate in perpendicular to the beam direction (Sarvazyan et al 1998) (figures 3(a)–(d)). In this study, to generate intense plane shear waves that covered a large wall thickness of the myocardial specimen, three focused beams were sequentially transmitted at different depths along the same axial axis inside the myocardium (figure 3(a)) (Bercoff
et al 2004). Generated shear waves, whose group velocity typically ranged between 1 and 10 m s\(^{-1}\) in soft tissues, were then imaged at an ultrafast frame rate between 8000 fps and 12 000 fps, which is more than 100 times higher than what conventional echocardiography scanners provide.

ETI achieves such ultrafast frame rates by transmitting acoustic plane waves while suffering from low echocardiographic signal-to-noise ratio (SNR) and thus degrading the tracking accuracy of the generated shear waves. Therefore, we further implemented the coherent plane-wave compounding technique (Montaldo et al 2009) to improve the echocardiographic image quality. In this study, shear waves were imaged by alternately steering ultrasonic plane waves at three different angles (-2°, 0°, 2°). Coherent-compounded images of the shear waves were formed by averaging three consecutive ultrasonic plane wave images. This moving-average procedure not only improved the image SNR but also preserved the initial ultrafast frame rate.

2.3.2. Data acquisition. A programmable prototype ultrasound system (Supersonic Imagine, Aix-en-Provence, France) equipped with a phased array probe (center frequency = 2.8 MHz, pitch = 0.28 mm, number of elements = 64, fractional bandwidth = 90%, pulse duration = 200 \(\mu\)s) was utilized. The ultrasound probe was firstly aligned (\(\beta = -90°\)) with the longitudinal axis (\(x_l\)) of the myocardial sample (figure 2(a)) and thereafter rotated counterclockwise to \(\beta = 90°\) at 5° increments. At each probe angle, plane shear waves were generated and acquired (figure 3(a)), and the total acquisition time was less than 10 ms.

A phased array probe was selected for the present study mainly because its smaller aperture size befitted the MRI scanner (see section 2.4). The phased array probe also enjoyed larger imaging depth. The myocardial sample was then placed 30 mm away from the surface of the probe with a gelatin phantom served as a standoff (section 2.2.1), so the artifact from the probe itself could be avoided and would not appear in the MR images. Furthermore, this phased array probe functioned as a linear array in order to generate plane acoustic transmissions for ultrafast imaging. According to Fresnel approximation for a rectangular aperture (Szabo 2004), the transition distance (\(z_t\)) of the phased array probe (operating at 4.5 MHz) used in this study was found to be 300 mm (\(z_t \approx \frac{L_x^2}{\pi c f} = \frac{182}{\pi (1.5/4.5)} \approx 300 \text{ mm}\), where \(L_x\) is the aperture size and \(c\) is the speed of sound). Throughout the experiments, imaging depth was up to 60 mm, and transmitted acoustic plane waves therefore presumably stayed plane at 60 mm.

2.3.3. Shear wave tracking and velocity estimation. At each probe angle, shear wave amplitude, namely tissue velocity resulting from the acoustic radiation force, was first estimated throughout the imaged myocardium using a standard speckle tracking method. One two-dimensional (2D) image of the shear wave amplitude was called a shear wave image (figure 3(b)). A time series of shear wave images allowed us to visualize the shear wave propagation (figures 3(b)–(d)). The analysis of the shear wave propagation could be implemented in the following steps.

First, at each depth of interest (1–1.5 mm), a spatio-temporal imaging of the shear wave propagation was obtained (figure 3(e)). Second, the wave propagation distance was estimated by tracking the wave profiles in successive frames using the standard normalized cross-correlation function (Lee et al 2012). Finally, the shear wave group velocity across the myocardial wall was therefore estimated in a depth-by-depth fashion. Since the rotation axis of the probe was centered at the middle of the probe, the myocardium at the central lateral position was the only region where shear wave propagations from all angles overlapped. Full
shear wave speed map as a function of myocardial wall depth and probe angles at the central lateral location was then reconstructed as shown in figure 3(f).

2.3.4. Fiber angle estimation. To map the fiber orientation, a total of 37 rotation angles (from $\beta = -90^\circ$ to $\beta = 90^\circ$ at every $5^\circ$) were realized (section 2.3.2), and each corresponding transmural shear wave speed was estimated to obtain the full shear wave speed map of the overlapped central lateral region (section 2.3.3). In our previous study (Lee et al 2012), the maximum-based strategy was performed and required a shear wave speed distribution as a function of both depth and all 37 angles (figure 3(f)). The estimated fiber angle values were a multiple of $5^\circ$ due to $5^\circ$ increments of the probe rotation. In other words, the estimated fiber angle precision was restricted to the rotation angle resolution of the ultrasound probe (i.e. shear wave propagation directions) applied. Even though interpolation (e.g., parabolic or spline) could have been exploited to improve the estimation precision, the shear wave speed distribution as a function of the rotation angle would have necessitated appropriate interpolation schemes. Different from the maximum-based strategy for fiber angle estimation presented in our previous study, a tensor-based approach which combined least-squares approximation and eigendecomposition was proposed as follows to circumvent the problem of the limited probe angle resolution.

In the linear elasticity theory, mechanical properties of a material can be described by the rank 4 stiffness tensor, $C$. The elastic wave propagation in a material can be described by (Royer and Dieulesaint 2000)

$$[C_{ijkl}n_in_k - \rho V^2 \delta_{ij}]P_i = 0,$$

where $n(n_1, n_2, n_3)$ and $P$ are the propagation and polarization directions, respectively, $V$ is the wave propagation group velocity, and $\delta$ is the matrix of Kronecker delta (i.e. an identity matrix). As shown in figure 2(a), we consider here the shear wave which is polarized along axis 3 (i.e. the radial axis, $r$) ($P = (0, 0, 1)$) and propagates in the $x_1-x_2$ (i.e. the circumferential-lateral) plane given by the propagation vector $n((n_1, n_2, 0))$ at a speed of $V_s$. Equation (1) then becomes

$$C_{3jk3}n_in_k - \rho V^2 s = 0 \quad (2)$$
$$C_{3jk1}n_in_k = 0 \quad (3)$$
$$C_{3jk2}n_in_k = 0. \quad (4)$$

Equations (2)–(4) can be expanded as

$$\begin{align*}
C_{3113}n_1^2 + C_{3223}n_2^2 + (C_{3123} + C_{3213})n_1n_2 &= \rho V^2 \\
C_{3112}n_1^2 + C_{3221}n_2^2 + (C_{3121} + C_{3211})n_1n_2 &= 0 \\
C_{3122}n_1^2 + C_{3212}n_2^2 + (C_{3112} + C_{3221})n_1n_2 &= 0
\end{align*} \quad (5)$$

Equations (3) and (4) describe relationships between the $C_{ijkl}$s which do not contribute to $V_s$ in this scenario and therefore must always hold, given the propagation direction $n((n_1, n_2, 0))$. Then, we obtain

$$C_{3111} = 0; C_{3221} = C_{3212} = 0; C_{3121} = C_{3112} = 0; C_{3211} = 0; C_{3222} = 0; C_{3122} = 0. \quad (6)$$

Equation (6) imposes the medium to have a certain minimum degree of symmetry for such a shear wave to propagate. With these six independent components equal to zero, the medium may possess orthotropic, tetragonal, hexagonal, cubic, or isotropic symmetry (Royer and Dieulesaint 2000). Shear wave propagation in equation (5) can be re-written as

$$C_{3113}n_1^2 + C_{3223}n_2^2 + 2C_{3123}n_1n_2 = \rho V^2 s. \quad (7)$$
For the propagation in the \(x_1-x_2\) (i.e. circumferential-longitudinal) plane, by substituting \(n_1 = \cos \beta\) and \(n_3 = \sin \beta\), where \(\beta\) is the probe rotation angle (i.e. propagation angle of the shear wave) (figure 2(a)), into equation (5), we have

\[
\rho V_s^2 = C_{3113} \cos^2 \beta + 2C_{3123} \cos \beta \sin \beta + C_{3223} \sin^2 \beta
\]

\[
= \begin{bmatrix} \cos \beta & \sin \beta \end{bmatrix} \begin{bmatrix} C_{3113} & C_{3123} \\ C_{3123} & C_{3223} \end{bmatrix} \begin{bmatrix} \cos \beta \\ \sin \beta \end{bmatrix}.
\]

Equation (8) can be further rewritten in the form of equation (2) as

\[
\vec{n}^T \mathbf{M} \vec{n} - \rho V_s^2 = 0,
\]

where \(\vec{n} = \begin{bmatrix} \cos \beta \\ \sin \beta \end{bmatrix}\) and \(\mathbf{M} = \begin{bmatrix} C_{3113} & C_{3123} \\ C_{3123} & C_{3223} \end{bmatrix} = \begin{bmatrix} \mu_{11} & \mu_{12} \\ \mu_{12} & \mu_{22} \end{bmatrix}\), which is the elastic tensor.

Equations (8) and (9) are valid for any \(\beta\) angle of the propagation direction, and the elastic tensor, \(\mathbf{M}\), can be solved using the least-squares method equation (11) based on \(N\) measurements of \(V_{\beta_i}\) (1 < \(i\) < \(N\)) for different angles of shear wave propagation equation (10):

\[
\rho \begin{bmatrix} V_{\beta_1}^2 \\ \vdots \\ V_{\beta_N}^2 \end{bmatrix} = \begin{bmatrix} \cos^2 \beta_1 & 2 \cos \beta_1 \sin \beta_1 & \sin^2 \beta_1 \\ \vdots & \vdots & \vdots \\ \cos^2 \beta_N & 2 \cos \beta_N \sin \beta_N & \sin^2 \beta_N \end{bmatrix} \begin{bmatrix} \mu_{11} \\ \mu_{12} \\ \mu_{22} \end{bmatrix} = \Phi \begin{bmatrix} \mu_{11} \\ \mu_{12} \\ \mu_{22} \end{bmatrix}
\]

with \(\Phi = \begin{bmatrix} \cos^2 \beta_1 & \sin 2 \beta_1 & \sin^2 \beta_1 \\ \vdots & \vdots & \vdots \\ \cos^2 \beta_N & \sin 2 \beta_N & \sin^2 \beta_N \end{bmatrix}_{N \times 3}^T\).

The least-squares solution of \([\mu_{11} \mu_{12} \mu_{22}]^T\) is obtained as

\[
\begin{bmatrix} \mu_{11} \\ \mu_{12} \\ \mu_{22} \end{bmatrix} = (\Phi^T \cdot \Phi)^{-1} \cdot \Phi^T \cdot \begin{bmatrix} \rho V_{\beta_1}^2 \\ \vdots \\ \rho V_{\beta_N}^2 \end{bmatrix}.
\]

In this study, myocardium is treated as an anisotropic medium as typically suggested for myocardial elasticity in the literature (Hunter et al. 2003, LeGrice et al. 1997). Consequently, the eigendecomposition of \(\mathbf{M}\) was finally performed (equation (12)) to obtain the elastic constants (\(\mu_{//}\) and \(\mu_{\perp}\)) in the orthotropic symmetry, whose principal axis is parallel to the \(x_2\)-axis (i.e. \(x_1\) in figure 2(a)):

\[
\mathbf{M} = \begin{bmatrix} \mu_{11} & \mu_{12} \\ \mu_{12} & \mu_{22} \end{bmatrix} = \mathbf{R} \begin{bmatrix} \mu_{//} & 0 \\ 0 & \mu_{\perp} \end{bmatrix} \mathbf{R}^T
\]

with \(\mathbf{R} = \begin{bmatrix} \cos \alpha & -\sin \alpha \\ \sin \alpha & \cos \alpha \end{bmatrix}\).

Shear waves that propagate along and across the fibers are further retrieved below:

\[
v_{//} = \sqrt{\frac{\mu_{//}}{\rho}}, \quad v_{\perp} = \sqrt{\frac{\mu_{\perp}}{\rho}},
\]

where \(v_{//}\) and \(v_{\perp}\) are speeds of the shear waves that respectively propagate along and across the fibers.
2.4. Diffusion tensor magnetic resonance imaging (DT-MRI)

2.4.1. Data acquisition. Immediately after the ETI acquisition, the myocardial sample along with the MR-compatible ETI setup (figure 2(b)) was then placed inside a 7T small bore MRI scanner (Bruker BioSpin, Germany) equipped with a 300 mT m\(^{-1}\) gradient insert and a radiofrequency volume coil of an inner diameter of 60 mm. A spin-echo-based diffusion sequence was used with six directions of diffusion encoding: (1, 0, 0), (0, 1, 0), (0, 0, 1), (\(\frac{1}{\sqrt{2}}, \frac{1}{\sqrt{2}}, 0\)), (0, \(\frac{1}{\sqrt{2}}, \frac{1}{\sqrt{2}}\)), (0, 0, \(\frac{1}{\sqrt{2}}\)). For each diffusion direction, 23–45 axial slice images (slice thickness = 0.5 mm, \(b = 1029\) s mm\(^{-2}\), FOV = 30 mm \(\times\) 30 mm, matrix size = 60 \(\times\) 64, 1.34 zero-fill acceleration factor, TR = 6750 ms, TE = 19.2 ms, 24 averages) were acquired depending on the size of the sample and the time allocation allowed for the scans. For most of the myocardial samples studied, a total of 270 diffusion-weighted images and 45 \(b_0\) images were acquired in approximately 14 h 30 min. Axial, coronal and sagittal T2-weighted RARE images (RARE factor 8, slice thickness = 0.5 mm, 50 slices, FOV = 30 mm \(\times\) 30 mm\(^2\), matrix size = 128 \(\times\) 96, 1.34 zero-fill acceleration factor, TR = 5822 ms, effective TE = 36 ms, 2 averages, acquisition time 2 min 20 s for each imaging orientation) were also acquired for inter-modality co-registration (section 2.5).

2.4.2. Tensor computation and fiber tracking. The diffusion-weighted images were analyzed using the DTI-track module provided in the software MedINRIA version 1.9.0 (Toussaint et al 2007). Diffusion tensors were firstly estimated (background removal threshold = 200; no smoothing), followed by fiber tracking (FA threshold = 300, smoothness = 20, minimum length = 10, sampling = 1) to reconstruct the myocardial fiber orientation throughout the myocardial wall.

2.5. Coregistration between ETI and DTI

The fiber orientation in the myocardial region which both ETI and DTI examined was compared thanks to the co-registered imaging system setup shown in figure 2(b). Volume-rendered axial, coronal and sagittal T2-weighted RARE images (RARE factor 8, slice thickness = 0.5 mm, 50 slices, FOV = 30 mm \(\times\) 30 mm\(^2\), matrix size = 128 \(\times\) 96, 1.34 zero-fill acceleration factor, TR = 5822 ms, effective TE = 36 ms, 2 averages, acquisition time 2 min 20 s for each imaging orientation) were also acquired for inter-modality co-registration (section 2.5).

2.6. Fractional anisotropy

In order to evaluate the degree of anisotropy in the myocardium, FA was computed. In DTI, FA for each sample point was obtained from the eigenvalues (\(\lambda_1, \lambda_2, \lambda_3\)) of the diffusion tensor (Tseng et al 2006) as follows:

\[
FA_{\text{DTI}} = \sqrt{\frac{3}{2}} \frac{\sqrt{(\lambda_1 + \lambda_2 + \lambda_3)^2 + 3(\lambda_1 - \lambda_2)^2 + 3(\lambda_2 - \lambda_3)^2 + 3(\lambda_3 - \lambda_1)^2}}{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}.
\]

(14)

In ETI, FA was defined based on the shear wave speed in orthogonal propagation directions using the following formula:

\[
FA_{\text{SWI}} = \sqrt{2} \frac{\sqrt{(v_{/\!\!/} - \overline{v})^2 + (v_{/\!\!/} - \overline{v})^2}}{\sqrt{v_{/\!\!/}^2 + v_{/\!\!/}^2}},
\]

(15)

\[
\overline{v} = \frac{v_{/\!\!/} + v_{/\!\!/}}{2},
\]

where \(v_{/\!\!/}\) and \(v_{/\!\!/}\) are derived from (9), and \(\overline{v}\) is the mean velocity, average of \(v_{/\!\!/}\) and \(v_{/\!\!/}\).
Figure 4. Transmural fiber orientation estimated by elastic tensor imaging (ETI) and diffusion tensor imaging (DTI) in the same porcine myocardial sample of the left-ventricular anterior region shown in figure 3: (a) ETI-estimated and (b) DTI-measured fiber orientations. The virtual fibers are oriented based on the estimated angles and color-coded. (c) The transmural fiber angle distributions estimated by both ETI and DTI. 0% and 100% wall depths indicate endocardium and epicardium, respectively. (d) The scatter plot of the ETI-estimated fiber angles against DTI-estimated fiber angles.

3. Results

The ETI- and DTI-estimated transmural fiber orientations of the same porcine myocardial sample presented in figure 3 are shown in figures 4(a) and (b), respectively. Virtual fibers were not only displayed but also color-coded according to the estimated angles at their respective wall depths. Transmural variation of the myocardial fiber orientation, approximately from 70° in the subendocardium (90% wall depth) to −50° in the subepicardium (20% wall thickness), was observed (figure 4(c)). Good correlation ($r^2 = 0.86$) between ETI and DTI was also found (figure 4(d)). Note that only partial myocardial wall depth was assessed from this sample owing to the limitation of DTI acquisition time allocated. Such time limitation also applied to two other myocardial samples shown later in this paper as noted in their transmural fiber distribution graphs. For one-to-one mapping of the fiber angle estimates, myocardial per cent...
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Figure 5. Transmural fiber distribution estimated by both ETI and DTI in (a) anterior, (b) lateral and (c) posterior wall regions of the left ventricle. 0% and 100% represent the endocardium and epicardium, respectively. (d)–(f) Corresponding scatter plots of the ETI-estimated fiber angles against DTI-estimated fiber angles shown in (a)–(c).

Three other myocardial samples dissected from the anterior (figure 5(a)), lateral (figure 5(b)) and posterior (figure 5(c)) regions of the left ventricle are shown. We again observed that the fibers orientated from positive (near the endocardium) to negative (near the epicardium) angles. Good correlation in fiber angle distributions between ETI and DTI estimation in each of the three samples was also shown (figures 5(d)–(f): \( r^2 = 0.95, 0.77 \) and \( 0.94 \), respectively). Transmural fiber angles estimated by ETI and DTI in all ten myocardial samples (or, a total of 295 comparison sites) studied were found to be well correlated (\( r^2 = 0.81 \)) (figure 6(a)) and in good agreement (bias: 3.05° and 95% limits of agreement: −30.63° to 36.73°) (figure 6(b)).

FA values were firstly computed (section 2.6) at each wall depth. The myocardial wall was then equally divided into three regions: subendocardium (0%–33% wall depth), midwall (33%–67% wall depth) and subepicardium (67%–100% wall depth). Average FA values were finally obtained in each region in each sample. The FA values computed from ETI and DTI measurements (figure 7) show that ETI exhibited higher average FA values than DTI in the subendocardial (0.51 versus 0.30, \( p < 0.02 \)), midwall (0.38 versus 0.29, \( p < 0.05 \), paired one-tailed t-test) and subepicardial (0.34 versus 0.27, \( p > 0.05 \)) regions. Paired, one-tailed t-test was performed for the comparison of FA values between ETI and DTI from ten samples in each region. On the other hand, unpaired, one-tailed t-test was performed for the comparison of FA values between two distinct wall depths from ten samples examined by each imaging technique. The average ETI-estimated FA values decreased from subendocardium to subepicardium (from 0.51 to 0.34, \( p < 0.05 \)) by 33%, whereas the corresponding DTI-estimated FA values presented a change of −10% (from 0.30 to 0.27, \( p > 0.05 \)). DTI-estimated FA values showed no significant decrease (\( p > 0.3 \)) from subendocardium (FA = 0.30) to midwall (FA = 0.29).
An in vivo examination of the myocardial fiber orientation using ETI was also performed on the mid-anterior region of a beating ovine heart. The fiber angle across this mid-anterior wall region was estimated at the midsystolic phase and mapped as shown in figure 8(a). The ETI-estimated transmural fiber orientation of this myocardial sample excised and situated in vitro is shown in figure 8(b). The transmural variation of the fiber angles estimated by ETI exhibited a similar trend (figure 8(c)) and a good correlation (figure 8(d); $r^2 = 0.89$) with that by DTI.

Given in equation (11), the ETI was immune from the $5^\circ$ step resolution of the rotation device and therefore yielded higher precision in estimated fiber angle values without an assumption of the shear wave speed distribution as shown in figures 4, 5 and 8. The effect of the number of probe angles (i.e. propagation directions) on the robustness of the tensor-based fiber angle estimation was further investigated. Seven scenarios where different numbers of
Map fiber orientation using ultrasound

Figure 8. Mapping myocardial fiber orientation of the apical-anterior wall region in one ovine left ventricle both (a) in vivo by ETI and (b) in vitro by ETI; (c) in vitro transmural fiber angle estimation by ETI and DTI, and (d) a scatter plot of ETI-estimated fiber angles against DTI-estimated fiber angles.

the rotation angles were inputted to solve equation (10) were tested in the myocardial sample shown in figures 3 and 4. In the 19-angle case, the shear wave data from the rotation angles ranging from -90° to 90° at every 10° were used. Similarly, rotation angles were sampled at every 15°, 20°, 30°, 45° and 60° to solve equation (10) in the 13-, 10-, 7-, 5- and 4-angle schemes, respectively. Figure 9 demonstrates that seven angles were deemed sufficient to provide transmural fiber orientation that was comparable to that estimated from 37 rotation angles.

4. Discussion

In this study, we have demonstrated that, for the first time, the myocardial fiber orientation estimated by ETI, which assesses the shear wave speed (and thus the shear modulus) in soft tissue, correlated well with that measured by DTI, which evaluates the water diffusion rate within the medium. Both ETI and DTI were performed in a total of ten in vitro myocardial samples, and ETI was further tested in one in vivo beating ovine heart. Moreover, in contrast to
Figure 9. The effect of the number of probe rotation angles used in ETI on the fiber angle estimation. The 37-angle case represents the scenario where rotation angles ranging from $-90^\circ$ to $90^\circ$ at five increments were used. Fiber distributions at (b) 0%–33%, (c) 33%–67% and (d) 67%–100% wall depth zones are identified by the orange dashed boxes in (a) and magnified.

Each myocardial sample possessed a different shear modulus from the surrounding gelatin. At the boundary, wave characteristics may have been changed. Different propagation modes might have been present and therefore complicated the true shear wave speed estimation. However, note that it was the anisotropy of shear wave propagation speed that determined the local fiber orientation, at which this present study aimed. As shown in figure 3(f), throughout the myocardial wall, shear wave speed anisotropy was prominent enough for the local fiber angle estimation thanks to the multiple propagation angles performed. In future studies where myocardial samples are prepared in the same fashion and where true mechanical properties
become essential, boundary conditions at such a solid–solid interface should be considered (Nenadic et al. 2011a).

The myocardial samples of $20 \times 20 \times 30$ mm$^3$ were embedded in cylindrical gelatin phantoms with a radius of 13.5 mm and a height of 60 mm. Throughout the experiments performed in this study, no reflections of shear waves from the myocardial edges were noted within the field of view (18 mm in the lateral range) and the transient propagation time window (3 ms) no matter whether the acoustic radiation force targeted at the left or the right side of the myocardial sample. This could be demonstrated in figures 3(c)–(e). Moreover, the full rotation of the probe allowed us to access only the overlapped wave propagation area, which was located at the central lateral position. Even if reflected waves existed, their propagation would not interfere with the forward shear waves at this overlapped lateral position during the transient events.

In general, very good correlation in the transmural change of myocardial fiber orientation was found between ETI and DTI (figures 4–6). Unlike DTI, which probed the microscopic tissue structure through water molecule diffusion, ETI assessed the macroscopic structure based on the local shear modulus, which was directly linked to shear wave propagation speed. A research group (McLean and Prothero 1992) has also demonstrated that there existed detailed differences in fiber orientation between the microscopic and macroscopic levels, albeit their general agreement. This may explain the slight disagreement in estimated fiber angles between ETI and DTI. Besides, in DTI, greater $b$ value may allow the diffusion in a longer scale, and the DTI acquisition sequence may be tuned to be sensitive to the same structural scale as ETI. However, the tradeoff between the sensitivity and SNR as a result of the $b$ value selected in DTI has an impact on the accuracy of the fiber tracking. Although discrepancies in fiber angle values between two imaging methods (an average difference of 3.05$^\circ$ in figure 6(b)) were noted, the estimated fiber angles spanned over a similar range. Furthermore, ETI demonstrated its capability of examining the fiber structure in three distinct (anterior, lateral and posterior) wall regions of the left ventricle, all of which exhibited transmural variation of the fiber directions (figure 5).

ETI and DTI were able to retrieve similar myocardial fiber arrangement in spite of their different inherent physical phenomena. This raised a question whether the degree of anisotropy, which could be quantified by FA (equations (10), (11)), was also comparable between two imaging methods. The bar plot of FA in figure 7 shows that averaged ETI-estimated FA values were higher than averaged DTI-estimated ones in all three myocardial wall regions. DTI measured water diffusion rate, which was related to the cellular arrangement; on the other hand, ETI estimated the shear wave speed, which was not only associated with the fiber orientation but also with the local tissue material properties (e.g., stiffness). In other words, DTI may have been sensitive to the structure only, while ETI was responsive to both fiber orientation and stiffness. Higher FA estimated from ETI than from DTI may thus have inferred that the stiffness contrast was more conspicuous than the water diffusion rate contrast inside the contracted myocardium assessed in our study.

Figure 7 also shows that averaged ETI-estimated FA decreased by 33% from the subendocardium to the subepicardium. Higher ETI-based FA in the inner myocardial zone than the outer part (figure 7) may have been related to local fiber mechanics. A study has shown increasing fiber stress from epicardium to endocardium (Bovendeerd et al. 1994). Costa et al. (2001) have summarized that fiber stiffness is generally 1.5–3 times greater than cross-fiber stiffness, that fiber stiffness increased from subepicardium to midwall while cross-fiber stiffness did not in the canine myocardium, and that there existed transmural inhomogeneity based on strain energy functions. This may have suggested that the fiber and cross-fiber stiffness ratio increased from subepicardium to midwall. The observation that the elasticity
anisotropy was non-uniform across layered myocardial wall was reflected on the heterogeneity in ETI-estimated FA. Our findings may imply that the transmural variation of diffusivity was less prominent than that of elasticity.

In this study, no significant decrease in DTI-estimated FA values was found from the midwall \((FA = 0.29)\) to subepicardial \((FA = 0.27)\) regions \((p > 0.05, \text{ unpaired, one-tailed} \ t\text{-test}, N = 10)\) and from midwall to endocardium \((p > 0.2, \text{ unpaired, one-tailed} \ t\text{-test}, N = 10)\). Such an observation contradicted the finding in a prior study \((\text{Jiang et al} 2007)\), which showed a relatively constant FA from subepicardium \((FA = 0.35 \pm 0.05)\) to midwall \((FA = 0.32 \pm 0.05)\) but a decrease from midwall to endocardium \((FA = 0.27 \pm 0.06)\) in their DTI measurements. In the study performed by Jiang et al \((2007)\), myocardial samples had been fixed in formalin for 4 ± 1 weeks before they were imaged with DTI, whereas in our experiments, freshly excised samples were embedded in the gelatin phantom without fixation for DTI scans. Furthermore, their mean FA values were obtained from all the myocardial segments from all five different short-axis levels of the left ventricle. As observed in figure 3A in Jiang et al \((2007)\), some FA profiles did not exhibit pronounced transmural variations. As listed in table 2 in Jiang et al \((2007)\), the slopes of the FA profiles (i.e. transmural changes in FA) in DTI varied with the myocardial segments. Our samples were excised from the short-axis level that approximately corresponded to the S3 left-ventricular level in their study. The FA values from this S3 level seemed to exhibit least transmural change. Whether transmural FA has a homogeneous or heterogeneous distribution should be further corroborated by measurements from \textit{in vivo} beating hearts, which exhibit physiologic cardiac mechanics.

Figures 8(a) and (b) respectively show the transmural distributions of myocardial fiber orientation estimated by ETI in the same ovine apical-anterior left-ventricular wall region under both \textit{in vivo} and \textit{in vitro} conditions. Although the same myocardial region was assessed, there existed physiologic discrepancies between \textit{in vivo} and \textit{in vitro} configurations. The \textit{in vivo} intact heart functioned in the presence of external loads (e.g., venous return and arterial pressure), while the isolated myocardial sample was load and stress free. Additionally, the excised heart studied was arrested in a contracture state, which may not have corresponded to a physiologic systolic phase in a cardiac cycle. The abovementioned phenomena may thus have resulted in the change of transmural fiber orientation before and after the excision of the heart. Despite currently unachievable \textit{in vivo} validation of ETI-estimated fiber orientation, the ETI-estimated \textit{in vitro} fiber angle distribution was well correlated with the DTI-estimated one (figures 8(c) and (d)) and demonstrated the \textit{in vivo} applicability of ETI.

Further tested in one myocardial sample and illustrated in figure 9, ETI was capable of reliably estimating fiber orientation through as few as seven rotation angles (equally sampled between \(−90°\) and \(90°\) at every \(30°\)), namely seven shear wave propagation directions. Each rotation angle required less than 10 ms acquisition time. When the rotation was implemented by a computer-controllable rotator, realizing real-time acquisitions could be feasible. Compared to our previous study, where all 37 rotation angles were used to find the fiber orientation, ETI entailed fewer shear wave propagation directions and a more efficient acquisition scheme while providing even higher fiber angle precision. In addition to higher precision and robustness in fiber angle estimation with the minimum number of rotation angles involved, the proposed tensor-based approach outperformed the maximum-based strategy in that it could simultaneously estimate the fiber angle \((\alpha)\) and the two elastic constants \((\mu//\text{ and } \mu\perp)\), given in equation \((9)\).

This proposed tensor-based approach has now shown the non-necessity of a full scale of rotation angles (figure 9) to retrieve the fiber orientations and might thereafter allow for efficient and accurate fiber angle mapping at multiple myocardial locations in the left ventricle, especially in the \textit{in vivo} non-invasive settings. Yet, the retrieved fiber structure is only one
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dimensional. Besides, the contribution of the laminar fiber architecture (i.e. sheet structure) to myocardial mechanics has been established (Costa et al 1999). Hence, mapping myocardial fiber architecture, including fiber and sheet structures, in 2D or 3D by ultrasound will be firstly resolved should a 2D ultrasound array become available.

The DTI performed in this study was programmed based on the standard DTI sequence compatible with the MRI scanner used. The selected parameters were a compromise among signal-to-noise ratio, spatial resolution and acquisition time. An echo planar imaging (EPI) accelerated sequence is currently being investigated to drastically reduce the acquisition time of DTI to fully access the entire myocardial sample without degrading the DTI quality.

Recall from the introduction that myofiber disorganization is prevalent in hypertrophic cardiomyopathy and post-infarction. Mapping the fiber orientation in such pathological myocardium to evaluate the clinical value of ETI is one of our future works. ETI may also be applied to other structured biological soft tissues, for example, brain and cornea, to study their material anisotropy.

5. Conclusion

In this study, we have proposed a novel ultrasound technique, elastic tensor imaging (ETI), to simultaneously estimate the shear moduli of the myocardium in the fiber and cross-fiber directions and to map transmural myofiber orientation through a tensor-based approach to solving elastic wave equations. We have also demonstrated that the precision of fiber angle estimation by ETI was no longer restricted to the propagation angular step size used and that a limited number of angle steps could be used. For the first time, ETI, which assesses the elastic anisotropy through shear wave propagation speed, was validated with magnetic resonance diffusion tensor imaging (DTI), which characterizes diffusion anisotropy based on the water diffusion rate, in mapping myocardial fiber structure. Results from ten normal in vitro myocardial samples show that the estimated fiber angles across the entire left ventricular wall depth were well correlated and agreed between ETI and DTI. Higher ETI-estimated FA values than DTI-estimated ones suggest that elastic anisotropy was greater than diffusion anisotropy in the normal myocardium. The feasibility of ETI in characterizing pathological myocardium in the presence of fiber disarray will be investigated.

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