Nano-imaging of the beating mouse heart in vivo: Importance of sarcomere dynamics, as opposed to sarcomere length per se, in the regulation of cardiac function

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Sarcomeric contraction in cardiomyocytes serves as the basis for the heart’s pump functions in mammals. Although it plays a critical role in the circulatory system, myocardial sarcomere length (SL) change has not been directly measured in vivo under physiological conditions because of technical difficulties. In this study, we developed a high speed (100–frames per second), high resolution (20-nm) imaging system for myocardial sarcomeres in living mice. Using this system, we conducted three-dimensional analysis of sarcomere dynamics in left ventricular myocytes during the cardiac cycle, simultaneously with electrocardiogram and left ventricular pressure measurements. We found that (a) the working range of SL was on the shorter end of the resting distribution, and (b) the left ventricular–developed pressure was positively correlated with the SL change between diastole and systole. The present findings provide the first direct evidence for the tight coupling of sarcomere dynamics and ventricular pump functions in the physiology of the heart.

INTRODUCTION

Myocardial sarcomeres undergo repeated cycles of shortening and lengthening in vivo, at a heart rate (HR) dependent on the animal species, and changes in lengths as small as ~100 nm dramatically change the heart’s pump functions (e.g., Allen and Kentish, 1985; Kentish et al., 1986; Kobirumaki-Shimozawa et al., 2014). Therefore, high speed, high resolution imaging of cardiac sarcomeres in vivo is paramount to fully understanding the contributions of sarcomere length (SL) dynamics to cardiac functions under physiological settings.

Recent advances in optical fluorescence technologies have enabled measurements of SL at high spatial and temporal resolution in cardiac cells by using various techniques such as AcGFP expression (i.e., SL nanometry; see Shintani et al., 2014, 2015), quantum dots (Serizawa et al., 2011), and fluorescence staining of the T-tubules (Bub et al., 2010; Botcherby et al., 2013; Ibarra et al., 2013; Inoue et al., 2013). Lee et al. (2012) imaged cardiomyocytes in vivo, as well as coronary arteries, in anesthetized open-chest mice at micron-level resolution, with relatively slow temporal resolution (i.e., one static image obtained during a single cardiac cycle at a fixed Z-direction in the microscope; see also Vinegoni et al., 2015). Recently, Aguirre et al. (2014) captured images of the fluorescence-labeled T-tubules in the mouse heart upon electric stimulation, via two-photon microscopy after reconstruction of the original images (see also Vinegoni et al., 2015). However, large 3-D movements of the heart per se, occurring under physiological settings as compared with skeletal muscle (e.g., Llewellyn et al., 2008, for sarcomere imaging in skeletal muscle in vivo using second harmonic generation), have hindered nano-scale analyses of SL displacement in cardiomyocytes in vivo under physiological settings. In addition, the similarity between the Z-disk spacing (i.e., SL) and T-tubular distance may not be maintained when the heart is at work, especially in a diseased condition (see Guo et al., 2013, and references therein). In the present study, therefore, we developed a high speed, high resolution sarcomere imaging system in the beating mouse heart in vivo and analyzed physiological sarcomere dynamics.
MATERIALS AND METHODS

This study was performed in accordance with the Guidelines on Animal Experimentation of The Jikei University School of Medicine. The study protocol was approved by the Animal Care Committee of The Jikei University School of Medicine and the Recombinant Gene Research Safety Committee of The Jikei University School of Medicine.

Adenovirus (ADV) infection in the heart in vivo

3–4-wk-old male BALB/c mice were anesthetized with ~2% isoflurane (Champion et al., 2003). Next, left thoracotomy was performed by using an electric scalpel (TCU-150; Geiger Medical Technologies) between the third and fourth ribs to visualize the anterior surface of the left ventricle (LV) under ventilation (200 µl at 300 per min). The animal was warmed at ~38°C (Bio Research Center Co., Ltd.). The ADV solution diluted by PBS (~) (viral titer, $10^{11}$–$10^{12}$ particles per ml) was injected into the epicardial surface of the central region of the LV (~5 µl/mm² in ~10 spots) by using a 1-ml syringe pump (MCIP-BO1; Minato Concept, Inc.) with a 32-gauge needle (Dentronics) under a stereoscopic microscope (SZ61; Olympus) (French et al., 1994; Fromes et al., 1999). One bolus injection of the ADV solution into the epicardial surface of the LV resulted stochastically in the expression of AcGFP in the Z-disks in approximately five cardiomyocytes per injected area. 2 d after chest closure, the mouse was anesthetized again with ~2% isoflurane and ventilated, and the anterior thoracic wall was removed by cutting the ribs, muscles, and intercostal arteries with the electric scalpel for in vivo cardiac sarcomere imaging. Because AcGFP expression efficacy tends to be higher in relatively young mice (3–4 wk) than in older ones (6–8 wk), we used 3–4-wk-old male BALB/c mice throughout the study.

In vivo sarcomere imaging and hemodynamic recording

The anesthetized open-chest mouse under ventilation was placed on a custom-made microscope stage (250 × 350 mm), and the animal was warmed at 38°C throughout imaging. Electrocardiogram (ECG) lead III was recorded by using an amplifier (JB-611J/MEG-6108/AB-611J; Nihon Kohden) (see Fig. 1, A and B). Likewise, the left ventricular pressure (LVP) was recorded by a catheter (FTH-1211B-0018; Transonic Systems Inc.) inserted from the apex of the heart (Champion et al., 2003). Diastolic LVP was not varied in the present study. A 488-nm laser was used in the confocal unit for the excitation of AcGFP expressed in the Z-disks of cardiomyocytes in the LV of the heart. The ECG and LVP signals and image acquisition timing from the EMCCD camera were simultaneously recorded by LabScribe software (iWorx Systems, Inc.) at 5 kHz. A coverslip (0.04–0.06-mm thick; no. 000; Matsunami Glass Ind., Ltd.) set on a metal ring (diameter, 12 mm) was gently attached at two points (~2 mm apart) to the LV surface with glue (diameter, ~2 mm) (see Fig. 1 B). This process was essential to minimize the local myocardial movements (i.e., to maximize the SL analysis resolution) in the targeted imaging region between the glue points. The position of the coverslip was carefully controlled by a custom-made micro-manipulator (Sigma Koki Co.), and then set at the position where the local myocardial movement was suppressed with little or no change in systolic LVP (decrease in systolic LVP of less than ~5%). The ventilator was turned off during imaging in the experiments shown in Fig. 1 (for ~10 s); however, it was turned on for the experiments with image sequence reconstruction (see below).

Isolated heart experiment

Experiments were performed as described in our previous study (Inoue et al., 2013). In brief, the heart was isolated from the mouse anesthetized with pentobarbital sodium (100 mg/kg, intraperitoneally), and perfused via the aorta with 5 ml of Ca²⁺-free HEPES-Tyrode’s solution containing 80 mM 2,3-butane-dione monoxime (BDM) at a speed of ~1 drop/s. Then, the heart was mounted on the custom-made microscope stage, and AcGFP-expressing Z-disks were imaged. In some experiments, CellMask Orange (5 µg/ml in the above solution, 2 ml at a speed of ~0.5
Figure 2. Fluctuation analysis for the length of a single sarcomere in a myocyte in an isolated perfused heart at rest. (A; top) Ventricular myocyte expressing α-actinin–AcGFP in Z-disks in the isolated heart at rest observed with a 40× lens (N/A, 0.80). (Bottom left) Time course of the change in SL. Imaging performed at 100 fps. (Bottom right) Histogram showing the variance of SL. SD (i.e., an index of single SL displacement resolution; see Serizawa et al., 2011; Shintani et al., 2014, 2015), 40 nm. Yellow arrow, sarcomere used for the SL analysis. (B; top) Ventricular myocyte expressing α-actinin–AcGFP in Z-disks in the isolated heart at rest observed with a 60× lens (N/A, 1.00). (Bottom left) Time course of the change in SL. Imaging performed at 100 fps. (Bottom right) Histogram showing the variance of SL. SD, 20 nm. In A and B, the hearts were perfused with Ca²⁺-free HEPES-Tyrode’s solution containing 80 mM BDM with little or no LV cavity pressure, hence in diastasis (Inoue et al., 2013). Yellow arrow, sarcomere used for the SL analysis.

Online supplemental material
Fig. S1 shows our cardiac nano-imaging system. Fig. S2 shows SL changes in the beating heart in vivo in a mouse with high HR. Fig. S3 shows changes in ΔLVP (difference in LVP in diastole and systole) and HR after thoracotomy. Fig. S4 shows sarcomeres in a myocyte in a beating heart in vivo obtained by using image sequence reconstruction. Fig. S5 shows cardiac sarcomere imaging with image sequence reconstruction (as in Fig. S4) in a mouse with high systolic LVP. Video 1 shows a ventricular myocyte in the beating heart in vivo without image sequence reconstruction. Video 2 shows a ventricular myocyte in the beating heart in vivo with image sequence reconstruction. Video 3 shows the myocyte in Video 2 but with no image sequence reconstruction. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201511484/DC1.

RESULTS
Fig. 1 A illustrates our in vivo cardiac sarcomere imaging system (see also Fig. S1, A and B). Hemodynamic parameters (i.e., ECG and LVP) were simultaneously recorded with real-time sarcomeric motions, and all signals were synchronized by a computer at 5 kHz (PC1 in Fig. 1 A). A 488-nm laser was used for excitation of AcGFP in the beating mouse heart (Fig. 1 A). Fig. 1 B illustrates the preparation of the animal for sarcomere imaging: ADV was injected into the epicardial surface of the LV. 2 d after the viral infection, the mouse was thoracotomized under anesthesia for sarcomere imaging. The ADV injection achieved effective expression of AcGFP in the surface of the LV (see green fluorescence in Fig. 1 C, left), and confocal observation revealed clear banding patterns along the longitudinal axis of the myocytes, indicating that AcGFP expression was localized in sarcomeric Z-disks (Fig. 1 C, right). See the Discussions in the supplemental text.

The AcGFP-expressing myocytes in the isolated perfused heart at rest are shown in Fig. 2. The precision values for the single SL displacement measurement...
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were 40 and 20 nm with objective lenses of 40× (N/A, 0.80) and 60× (N/A, 1.00), respectively (Fig. 2, A and B). These values were considered to be sufficiently small enough to quantify SL displacement at ~100-nm levels (see Serizawa et al., 2011; Shintani et al., 2014, 2015).

The experiments based on AcGFP expression using ADV (as well as the fluorescence agent CellMask for staining the T-tubules; see Ibarra et al., 2013; Inoue et al., 2013) in the isolated heart at rest revealed that the resting SL value was 1.97 ± 0.20 µm in the LV (compare Chung and Granzier, 2011) (Fig. 3 A; see Fig. 3, B and C, for the similarity between SL and the T-tubular distance).

We found that sarcomeres regularly repeated shortening and lengthening in synchronization with the movement of the myocyte per se in the beating heart in vivo (Video 1). Fig. 4 A summarizes the typical time-dependent changes in SL in the myocyte during one cardiac cycle. The sarcomeric contraction consisted of shortening followed by relatively slow lengthening, as demonstrated by Iribe et al. (2007) in isolated guinea pig ventricular myocytes in the physiological work-loop style contraction. Multi-peak Gaussian fitting provided the SL values of 2.00 ± 0.11 and 1.72 ± 0.07 µm (n = 5 sarcomeres, as indicated by yellow rectangles in the images in Fig. 4 B, top) in diastole (i) and systole (ii), respectively (hence the shortening ratio, ~14%). And SL was lengthened to 1.97 ± 0.10 µm in subsequent diastole (iii). Despite the fact that measurements were made in the same myocyte during the cardiac cycle, SL varied by a magnitude of ~300 nm in both diastolic and systolic phases (see Sarai et al., 2002, for SL variance in isolated rat cardiomyocytes), which is greater than the precision value of 40 nm for SL displacement under the experimental setting (see Fig. 2 A). It should likewise be pointed out that the image was defocused in systole because of the movement of the myocyte along the Z-axis (Fig. 4 B).

Fig. 4 C shows the average values of five consecutive SLs from 29 cardiac cycles obtained from the animal used for Fig. 4 (A and B). Here, we refined the analysis method by developing software based on the two-peak Gaussian fitting, with the aim of quick acquisition of the

![Figure 3](https://example.com/figure3.png)

**Figure 3.** SL variance in myocytes in isolated perfused hearts at rest. (A; top) Typical confocal image of a myocyte expressed with AcGFP in Z-disks via ADV injection. Same as in Fig. 1 C, right. Sarcomeres in the yellow rectangular outline were used for the analysis in the middle trace. Bar, 10 µm. (Middle) Plot profile in the yellow rectangular outline in top. SL, 1.97 ± 0.22 µm. (Bottom) Frequency plot showing SL in myocytes expressed with AcGFP. Data fitted by a single Gaussian function (peak value, 1.97 ± 0.20 µm). Number of sarcomeres analyzed, 6,281 (in 50 myocytes from three hearts). The hearts were perfused with Ca²⁺-free HEPES-Tyrode’s solution containing 80 mM BDM (hence in diastasis; see Inoue et al., 2013), and the myocytes were observed by using appropriate emission filters (see Materials and methods). (B) Striation patterns with AcGFP expression. (Top) Typical confocal image of a myocyte expressed with AcGFP in Z-disks via ADV injection. After isolation, the heart was treated with CellMask according to our previously published procedure (Inoue et al., 2013). Note clear striation patterns along the myocyte (compared with those with CellMask treatment in C). Sarcomeres in the yellow rectangular outline were used for the analysis in the middle trace. Bar, 10 µm. (Middle) Plot profile in the yellow rectangular outline in top. Striation spacing distance (i.e., SL), 1.81 ± 0.13 µm (P > 0.05 compared with the data in B, middle). (Bottom) Frequency plot showing striation spacing distance in myocytes treated with CellMask. Data fitted by a single Gaussian function (peak value, 1.81 ± 0.29 µm). Number of sarcomeres analyzed, 307 (in seven myocytes from two hearts). (C) Striation patterns with CellMask treatment. (Top) Typical confocal image of a myocyte stained with CellMask at the T-tubules (Inoue et al., 2013). Bar, 10 µm. (Middle) Plot profile in the yellow rectangular outline in top. Striation spacing distance, 1.82 ± 0.13 µm (P > 0.05 compared with the data in B, middle). (Bottom) Frequency plot showing the variance of striation spacing distance in myocytes treated with CellMask. Data fitted by a single Gaussian function (peak value, 1.81 ± 0.29 µm). Number of sarcomeres analyzed, 307 (in seven myocytes from two hearts). In both B and C, the hearts were perfused with Ca²⁺-free HEPES-Tyrode’s solution containing 80 mM BDM. And the AcGFP-expressing hearts were treated with CellMask to compare fluorescence intensity between AcGFP and CellMask. The images in B and C were obtained from a different myocyte than that in A.
peak diastolic and systolic SL values (see Materials and methods in the supplemental text for details). As shown in Fig. 4 D, this fitting provided maximal (diastolic) and minimal (systolic) SL values with great ease, from the data of multiple cardiac cycles (i.e., 1.93 and 1.73 µm for maximal and minimal values, respectively, from 29 cardiac cycles in the animal used for Fig. 4 C). Accordingly, experiments using 13 mice demonstrated that the SL values averaged 1.90 ± 0.06 and 1.68 ± 0.06 µm in diastole and systole, respectively (i.e., average shortening ratio, ~12%) (Fig. 4 E). It can therefore be said that under physiological settings in vivo, the working SL range is within the shorter side of the resting SL distribution (i.e., 1.97 ± 0.20 µm; see Fig. 3 A).

We found a significant positive correlation between SL (ΔSL; difference in SL in diastole and systole) and peak diastolic and systolic SL values (see Materials and methods in the supplemental text for details). As shown in Fig. 4 D, this fitting provided maximal (diastolic) and minimal (systolic) SL values with great ease, from the data of multiple cardiac cycles (i.e., 1.93 and 1.73 µm for maximal and minimal values, respectively, from 29 cardiac cycles in the animal used for Fig. 4 C). Accordingly, experiments using 13 mice demonstrated that the SL values averaged 1.90 ± 0.06 and 1.68 ± 0.06 µm in diastole and systole, respectively (i.e., average shortening ratio, ~12%) (Fig. 4 E). It can therefore be said that under physiological settings in vivo, the working SL range is within the shorter side of the resting SL distribution (i.e., 1.97 ± 0.20 µm; see Fig. 3 A).

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LVP (ΔLVP; difference in LVP in diastole and systole) within the range of ΔSL from ~0.1 to ~0.4 μm (Fig. 5 A), providing direct evidence that changes in SL at ~100-nm levels significantly affect the heart’s pump functions. When ΔLVP was plotted against the percent SL shortening ratio, a significant correlation was likewise observed (Fig. 5 B). However, no correlation was found between diastolic SL or systolic SL and ΔLVP (Fig. 5, C and D), suggesting that ΔSL (or SL shortening ratio) is the most reliable index reflecting myocardial contractility in vivo. HR varied between ~250 and ~550 beats per minute (bpm) in the present study; however, no significant correlation was found with ΔLVP (Fig. 5 E).

Fig. S2 shows an α-actinin–AcGFP–expressing myocyte in a mouse showing the highest HR value in Fig. 5 A. Each plot was obtained from one animal with the average within the range of LVP; difference in LVP in diastole and systole (LVP; see Fig. 5 A–D). However, no significant correlation existed between parameters. (E) SL shortening ratio (%) plotted against LVP. No significant correlation existed between parameters. (D) Systolic SL plotted against LVP. No significant correlation existed between parameters. Number of animals, 10. In all panels, gray symbols indicate the data obtained from the animal in Fig. 4, A–D.

Conversion of the data allowed us to obtain an SL–LVP relationship, clearly showing in vivo that LVP increased (or decreased) upon SL shortening (or lengthening) (Fig. 6 D). As shown in Fig. 6 E, SL was shorter at the peak of systole (phase 0) than in diastole (phase ~8), indicating clear contractile motions of sarcomeres during consecutive cardiac cycles within the shorter range (i.e., 523 bpm). Striations were clearly observed in both systole and diastole, with distinct SL shortening, indicating the capability of our imaging system to derive SL values from mice with various HR values. It is likewise worthwhile noting that our imaging system is potentially applicable to animals with a more rapid HR of 600–700 bpm, because of the imaging rate (i.e., 100 frames per second [fps]) being ~10 times higher than these values.

In Fig. 4 E, one mouse exhibited extraordinarily short SL values (1.50 and 1.38 μm in diastole and systole, respectively). We conclude that because the mouse was under relatively deep anesthesia with isoflurane, there were exaggerated cardiodepressant effects (see Jensen et al., 1992; Kanaya et al., 1998) after thoracotomy. Indeed, the result of another set of experiments without imaging demonstrated that after thoracotomy, ΔLVP decreased significantly, whereas the HR remained nearly unchanged (Fig. S3).

We then developed a digitally controlled image-reconstruction system. Namely, the position of the objective lens (hence the focal point) was moved downward in the Z-direction at a 1-μm increment by using the piezo actuator (PC2 in Fig. 1 A; see Fig. 6, A and B). After experimentation, the LVP record was divided into 17 phases (i.e., 10.2 ms per phase; indexed from ~8 to 8, with the zero point set at its peak); best-focused images were selected at each phase and then combined to obtain an image sequence (Video 2). Video 2 shows clear sarcomeric motions during beating, compared with the original movie obtained in the same animal without image sequence reconstruction (Video 3). As confirmed by the plot profile analysis (Fig. S4, A–D), the images were consistently in focus during the cardiac cycle. Our method likewise revealed that the cardiomyocytes in the epicardial surface of the LV continuously moved in the Z-direction by a magnitude of ~5 μm during the cardiac cycle (Fig. S4, E and F).

Fig. 6 C summarizes the time course of changes in SL and LVP during the cardiac cycle, obtained by our image-reconstruction system (the myocyte located in the center of the LV). It was found that LVP started to develop (or fall) upon shortening (or lengthening) of SL. The SL shortening ratio was 8% in this mouse, slightly smaller than the average value (i.e., ~12%) in Fig. 5 B. We consider that the lesser magnitude of the ratio is caused by the relatively low level of systolic LVP in this particular mouse (i.e., ~40 mmHg in systole; see Fig. 5 with a variance of ΔLVP from ~40 to ~100 mmHg). Conversion of the data allowed us to obtain an SL–LVP relationship, clearly showing in vivo that LVP increased (or decreased) upon SL shortening (or lengthening) (Fig. 6 D). As shown in Fig. 6 E, SL was shorter at the peak of systole (phase 0) than in diastole (phase ~8), indicating clear contractile motions of sarcomeres during consecutive cardiac cycles within the shorter range.
DISCUSSION

In this study, we performed SL nanometry on left ventricular myocytes in living mice, and demonstrated a tight coupling between sarcomere dynamics and ventricular pump functions. Here we discuss the present findings, focusing on the role of sarcomere dynamics in the modulation of ventricular pump functions in vivo.

First, a positive correlation existed between $\Delta SL$ (or SL shortening ratio) and $\Delta LVP$ (Fig. 5, A and B), but not between diastolic (or systolic) SL and $\Delta LVP$ (Fig. 5, C and D). These findings suggest that when data are collected from different individuals, $\Delta LVP$ is related to the magnitude of SL change (i.e., $\Delta SL$), at least in the epicardium of the LV, more strongly than to the absolute diastolic SL values in vivo. Consistent with the present findings, a previous study reported no significant correlation between the SL shortening ratio and the absolute resting (=initial [diastolic]) SL in ventricular myocytes isolated from mice (data obtained from 17 mice) (Pohlmann et al., 2007). We consider that these sets of data obtained under different experimental conditions (i.e., in vivo and in myocytes) do not contradict the Frank–Starling law of the heart (see Katz, 2002; Kobirumaki-Shimozawa et al., 2014, and references therein), because the variances in the initial SL and myocardial contractile performance obtained from different individuals are inevitable, and they tend to mask the correlation between the diastolic SL and cardiac contractility.

The positive relationship between $\Delta SL$ and $\Delta LVP$ may have novel mechanistic implications regarding the relation between the amount of Ca$^{2+}$ released during the Ca$^{2+}$ transient (CaT) and thin filament activation (and the ensuing cross-bridge formation) in myocardium.
under auxotonic conditions, as in the beating heart in vivo. It is well established that the activation of thin filaments during the CaT is submaximal, and the occupancy of thin filament sites by myosin molecules never achieves a steady state (e.g., Bers, 2001; Kobirumaki-Shimozawa et al., 2014, and references therein). Therefore, given the brief duration of the CaT (see Tallini et al., 2006, for the CaT in the beating heart), the amount of Ca\textsuperscript{2+} released during the CaT (which is related to the duration of the CaT), rather than the peak value of the CaT, may determine the fraction of the thin filament that switches from the “off” state to the “on” state and thus the kinetics of contraction caused by the activation dependence of the cross-bridge cycling rate (e.g., Kobirumaki-Shimozawa et al., 2014, and references therein). Indeed, it has been reported that the amount of Ca\textsuperscript{2+} released during the CaT affects the on–off equilibrium of the thin filament state and the ensuing cross-bridge cycling rate in myocardium under physiological submaximal activation (e.g., Fitzsimons and Moss, 2007; Moss and Fitzsimons, 2010). Accordingly, it is likely that cross-bridge attachment during the course of the CaT (i.e., sarcomere shortening) can further recruit cross-bridge binding via thin filament cooperative activation, resulting in a positive correlation between ASL and cardiac contractility (i.e., ΔLVP; as in Fig. 5 A). We conclude that this cross-bridge–dependent mechanism is relatively stable across species and hence appears in the data from a variety of animals in vivo, because of the constant determinants of myofibrillar contractility across species (e.g., affinity of troponin C for Ca\textsuperscript{2+}, the rate of cross-bridge cycling, and propagation of thin filament cooperative activation via bound cross-bridges). To fully uncover the molecular mechanisms by which the initial SL or the magnitude of SL change (or both) determines cardiac contractility in vivo, it is necessary to simultaneously analyze the ventricular function and Ca\textsuperscript{2+}-dependent sarcomere dynamics in various layers of the heart (i.e., epicardium, midwall, and endocardium) in the same individual mouse at varying levels of ventricular filling by developing high speed, high performance two-photon microscopy. Along this line, it is of importance to investigate in future studies the effects of β-adrenergic stimulation on the relation between ASL and ΔLVP (or between diastolic/systolic SL and ΔLVP) in various layers of the ventricle.

The time-dependent changes in SL and LVP, regardless of the level of LVP (Figs. 6 C and S5 A), are qualitatively in good agreement with the findings of previous studies using synchrotron x-ray (Pearson et al., 2004, 2007; Shirai et al., 2013). These researchers applied the x-ray technique to the whole heart by using a high energy source, and developed a new cardiac-imaging technology in that the changes in the intensity ratio (I\textsubscript{00}/I\textsubscript{11}; the ratio of thick filament density to thin filament density) in the epicardial surface of the LV were obtained. It was accordingly found that during the cardiac cycle, I\textsubscript{00}/I\textsubscript{11} and LVP changed in a reciprocal manner in healthy anesthetized open-chest rats. Moreover, this is reportedly the case for induced pluripotent stem cell–derived cardiomyocytes planted in the epicardial surface of the LV in the anesthetized nude rat (Higuchi et al., 2015). Therefore, the present findings, combined with these lines of previous evidence, support the notion that in the epicardial surface of the central part of the LV wall in rodents, LVP increases (decreases) when sarcomeric contraction commences (terminates) according to the cellular level of the excitation–contraction coupling. The time point for peak SL shortening and that for peak LVP may deviate in other regions, such as in the base or apex, for the ventricle to efficiently eject blood.

Chung and Granzi (2011) reported that passive SL varied in a location-dependent manner in the isolated mouse heart upon barium chloride–induced contraction; SL varied as follows at maximal developed pressure versus no preload (in µm): 2.08 ± 0.01 versus 1.86 ± 0.01, 2.04 ± 0.01 versus 1.80 ± 0.01, and 1.98 ± 0.02 versus 1.73 ± 0.02 in the epicardium, midwall, and endocardium, respectively. We therefore consider that under physiological settings at high heartbeat frequencies in mice in vivo, the working SL range is shifted toward the shorter SL distribution side, presumably as a result of repeated myofibrillar shortening with a short diastolic filling period (thereby causing the difference in the SL measurement in the resting isolated heart and in vivo; compare Figs. 5 A and B). Likewise, because of the nature of the Nipkow confocal scanner, our in vivo cardiac-imaging system enables visualization of the sarcomeric motions in myocytes in the epicardial surface of the ventricle (i.e., restricted to a depth of ~150 µm because of optical limitations). Therefore, the SL values in myocytes elsewhere (e.g., deeper in the LV wall) may differ from those obtained in the present study in vivo (as reported in Chung and Granzi, 2011).

Because previous studies on cardiac imaging have defined the T-tubular spacing as SL (Bub et al., 2010; Botcherby et al., 2013; Inoue et al., 2013; Aguirre et al., 2014; Vinegoni et al., 2015), we directly compared the Z-disk spacing (i.e., SL) and T-tubular spacing in the present study, and found that SL and the T-tubular spacing showed similar values in the isolated heart at rest (Fig. 3). However, careful consideration must be given, because this similarity may not hold true when the heart is at work. Likewise, the T-tubular geometry relative to the Z-disks may be altered in failing hearts upon structural changes in the T-tubular system (Guo et al., 2013), limiting the application of “T-tubular imaging” in disease models. Moreover, because of the lack of the T-tubular network (Dibb et al., 2013), T-tubular imaging cannot be applied to atrial muscle. Therefore, our real-time nano-imaging of the Z-disks...
via expression of α-actinin–AcGFP has a high potential in elucidating the molecular mechanisms of cardiac sarcomere contractility in vivo under various conditions. The image acquisition became difficult in mice with high systolic LVP, primarily because of the movement of the target myocyte along the Z-direction in association with the heartbeat. However, despite that difficulty, the results of mice with low (Fig. 6 C) and high (Fig. 5 A) LVP values suggest that our image-reconstruction system will be useful in the systematic analysis of sarcomere dynamics in mice with various afterload resistance levels, e.g., in disease models.

In conclusion, our cardiac nano-imaging system has yielded direct evidence for the tight coupling between sarcomere dynamics and ventricular contractile function in vivo. The mechanistic implication is that under physiological conditions, the amount of Ca²⁺ released during the CaT is likely to activate thin filaments to a level where myocardial active force is determined by the rate of cross-bridge attachment via continuous thin filament cooperative activation by bound cross-bridges. At relatively high levels of activation, the kinetics of contraction would be expected to be near maximal, as the cross-bridge cycling rate is near maximal (e.g., Moss et al., 2004, and references therein). Thus, in mice, the Frank–Starling mechanism would not be particularly evident, which is the case in the present study. However, it is likely that the Frank–Starling mechanism contributes to a much greater degree in myocardium from larger animals, including humans, as the activation state during a twitch is much less than maximal and thus the cross-bridge cycling rate is lower. Future studies should be designed to systematically and thoroughly investigate the means by which the changes in SL in myocytes in various regions and layers of the myocardium modulate the heart’s pump functions in health and disease.

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Author contributions: F. Kobirumaki-Shimozawa, K. Oyama, T. Terui, S. Minamisawa, S. Ishiwata, and N. Fukuda designed the research. F. Kobirumaki-Shimozawa, K. Oyama, and T. Shimoza developed the microscopic system. T. Shimozawa wrote software. F. Kobirumaki-Shimozawa and T. Ohki designed ADV. F. Kobirumaki-Shimozawa, K. Oyama, and A. Mizuno performed experiments and analyzed the data. F. Kobirumaki-Shimozawa, K. Oyama, T. Shimozawa, and N. Fukuda wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Supplemental Materials and methods

Vector construction
Recombinant ADVs encoding mouse α-actinin-3-AcGFP (Ad-α-actinin-3-AcGFP; GenBank accession no. NM_013456) were constructed by using the AdMax ADV vector creation kit (Microbix Biosystems Inc.) as suggested by the manufacturer. The α-actinin-3 gene was amplified by using PCR with primers designed according to α-actinin-3 cDNA. Then, the α-actinin-3 cDNA was subcloned to pAcGFP-N1 (Takara Bio Inc.) by using Xhol and Asp718. The PCR fragments were synthesized by PCR using the primers fm_NheI-actinin (5′-ATGCTAGCACCATGATGATGGT-TATGCAGCCC-3′) and rm_AcGFP-SalI (5′-ATGCTAGCACCATGATGATGGT-TATGTCGACT-3′), and cloned into pDC215 entry vector (NheI/Sall). The pDC215-α-actinin-3-AcGFP entry vector and the ADV genome plasmid (pBHCgres1,5FLP) were co-transfected into HEK 293 cells (included in the AdMax ADV vector creation kit) with Lipofectamine LTX (Life Technologies). The cells were overlaid on DMEM medium containing 10% bovine serum and 1% agarose, and incubated at 37°C (±5% CO2).

Purification of ADVs
The ADVs were purified by using the Vivapure AdenoPack 20 purification kit (Sartorius AG) for injection into the LV of the heart in vivo. The elution buffer containing the purified ADVs was replaced with PBS (−) (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.4). Then, the ADVs were concentrated by ultrafiltration to yield 1011–1012 viral particles per ml in PBS (−) using VIVASPIN 15R (Sartorius AG) for 40–50 min at 3,000 rpm. The viral titer was calculated from the absorbance at 260 nm in the 0.1% (wt/vol) SDS solution (containing 10 mM Tris and 1 mM EDTA).

Microscopic system for imaging
An upright microscope (BX-51WI; Olympus) combined with a Nipkow confocal scanner (CSU21; Yokogawa Electric Co.) and an electron-multiplying CCD (EMCCD) camera (iXon384; Andor Technology) were used based on our previously published procedure (Inoue et al., 2013), at a 512 × 170-pixel resolution at a 100-Hz frame rate throughout the study. A water-immersion lens, either 40× (LUMPlanFL N 40 × W; N/A, 0.80; Olympus) or 60× (LUMPlanFL N 60 × W; N/A; 1.00; Olympus), was used for cardiac sarcomere imaging. A 2× lens (XLFluor 2×/340; N/A, 0.14; Olympus) was used to check AcGFP expression in the whole area of the LV. A custom-made swivel base (Lucir Inc.) was placed between the confocal scanner and EMCCD camera to adjust the orientation of a myocyte (i.e., AcGFP-expressing Z-disks perpendicular to the longitudinal axis of the view window). AcGFP-expressing myocytes in the heart were excited by a 488-nm laser light (HPU50211-PPS; Furukawa Electric Co.) that was directly introduced into CSU21 via air propagation. The diameter of the laser beam was controlled by a beam expander consisting of a concave and a convex lens pair in the optical path to adjust the excitation radius at the sample plane, and the beam was focused at the fiber port of CSU21. Accordingly, the resultant fluorescence signals (emission filter, BA510–550; Olympus) were detected. The usefulness of the in vivo nano-imaging microscopic system for anesthetized mice has already been demonstrated by others in the research areas of cancer (Tada et al., 2007; Gonda et al., 2010) and peripheral circulation (Hamada et al., 2011). By counting the number of myocytes in the Z-direction, it was shown that our optics system with the Nipkow confocal unit enabled imaging at the aforementioned spatial and temporal resolution at a depth of up to ~150 µm in the heart in vivo.

SL analyses
SL was measured by analyzing the fluorescence plot profiles along the longitudinal axis of an AcGFP-expressing myocyte by using ImageJ software (the region of interest [ROI], 5 pixels; National Institutes of Health). In brief, the profiles were analyzed using the multi-peak Gaussian fitting with a linear function of offset (Y = aX + b), based on the Levenberg–Marquardt algorithm, and the lengths of individual sarcomeres were calculated as the distance between the centers of two adjacent peaks (indicating the positions of the Z-disks; Serizawa et al., 2011; Shintani et al., 2014, 2015). The position of the ROI was adjusted in each image to enable accurate measurement of the length of the same sarcomere throughout the course of the cardiac cycle. To minimize the error in the SL analysis, sarcomeres showing insufficient fluorescence intensity (FL) in Z-disks were not included in the data. Likewise, we developed software to expedite the analysis of diastolic and systolic SL: A frequency histogram was plotted against SL based on the data from a myocyte during ~50 cardiac cycles (300–500 frames used from one myocyte; see Fig. 4 C). Then, the histogram was fitted with two Gaussian functions using the cumulative frequency plot to obtain binning-independent fitting results (see below for details). The diastolic (or systolic) SL was defined as the greater (or lesser) peak position (see Fig. 4 D). In experiments with isolated hearts, SL or the T-tubular distance (i.e., distance between two fluorescence peaks with CellMask Orange) was measured from static images by multi-peak Gaussian fitting (Fig. 3).

Two-peak Gaussian fitting of SL
Frequency distribution plot for SL (mean of five sarcomeres) provided two peak positions independent of the binning width. The curve fitting was performed on the cumulative probability plot of the SL histogram using the combination of two integrals of the normalized Gaussian functions, i.e.:

\[
f(x) = \frac{A_1}{\sqrt{2\pi}\sigma_1^2}\exp\left(-\frac{(x-\mu_1)^2}{2\sigma_1^2}\right) + \frac{1-A_1}{\sqrt{2\pi}\sigma_2^2}\exp\left(-\frac{(x-\mu_2)^2}{2\sigma_2^2}\right),
\]

where \(\mu\) and \(\sigma^2\) (with subscript numbers) indicate peak positions and distribution variances, respectively. \(A_1\) was set as 0 ≤ \(A_1\) ≤ 1 in the present study. Fitting the frequency distribution plot with this function provided diastolic and systolic SL values as 1.93 ± 0.03 and 1.73 ± 0.07 µm, respectively (see Fig. 4 D).

Effects of thoracotomy on LVP and HR
First, in the 3–8-wk-old BALB/c male mouse anesthetized with ~2% isoflurane, the abdomen was opened using an electric scalpel (see above), and a small incision (diameter, ~3 mm) was made in the diaphragm using the scalpel. Then, a catheter (see above) was inserted into the LV through the apex of the heart,
and LVP and ECG were monitored throughout experimentation at 5 kHz under ~2% isoflurane according to the method described above. After LVP was measured for ~20 s under the steady-state condition, the mouse was thoracotomized, and LVP was measured again for ~20 s. Data obtained during one observation, before and after thoracotomy, were averaged for LVP and HR.

Supplemental Discussion

Supplemental Discussion 1

The Nipkow confocal system has been widely used for the imaging of fast-moving objects; however, this system has an intrinsic “pinhole cross-talk” problem (Shimozawa et al., 2013). Namely, fluorescence in defocused image planes passes through neighboring pinholes in the Nipkow disk, thereby generating high background noise. This intrinsic problem appeared in the present study as the low contrast image in the CellMask-labeled myocyte (Fig. 3 C). Therefore, we used ADV and imaged AcGFP-expressing myocytes (surrounded by non-expressing myocytes) to obtain high contrast images.

Supplemental Discussion 2

The myocyte relative to the confocal image plane may result in an overestimation of SL. However, taking into consideration the careful analysis by the group of Bub (Bub et al., 2010; Botcherby et al., 2013) combined with our SL analysis based on the Gaussian fitting, the maximal overestimation would be ~3% in the present study, because the length of in-focus images in the image plane (i.e., \(L_{\text{min}}\) in Bub et al., 2010, and Botcherby et al., 2013) was longer than ~35 µm in all experiments performed (as in, e.g., Figs. 4 B and 6 B). Moreover, our image reconstruction system can maintain \(L_{\text{min}}\) at a similar level during the cardiac cycle with a variance of less than ~10% (see Figs. 6 B, S4, and S5). It is therefore safe to consider that the impact of the myocyte angle on SL is nearly negligible in the present experimental settings.

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S2 Cardiac sarcomere nano-imaging in vivo
Figure S1. In vivo cardiac sarcomere imaging system. (A) The microscopic system consisting of a Nipkow confocal unit, an EMCCD camera, and a piezo objective lens positioner. A custom-made swivel base was placed between the confocal scanner and EMCCD camera to adjust the orientation of myocytes in the view window (see Fig. 1A). The emission filters were placed between the confocal scanner and swivel base. (B) Close-up view of the stage of the microscope. The piezo unit was placed on the base of the objective lens (see Fig. 6 and Materials and methods). The micro-manipulator on the left was used to hold the metal objective ring described in Fig. 1A. Similarly, the micro-manipulator on the right was used to hold the metal guide for the LVP catheter.

Figure S2. SL changes in the beating heart in vivo in a mouse showing high HR. (Top left) Confocal image of the myocyte in systole. Sarcomeres numbered from 1 to 7 were analyzed. (Bottom left) Plot profile along the longitudinal axis of the myocyte in top. SL, 1.75 ± 0.04 µm. (Top right) Confocal image of the myocyte in diastole. Sarcomeres numbered from 1 to 7 were analyzed (i.e., same as in left) because the image was in focus in this animal. (Bottom right) Plot profile along the longitudinal axis of the myocyte in top. SL, 1.99 ± 0.07 µm (P < 0.05 compared with the value in systole). Note clear striations in both systole and diastole at high HR. Measurements were made on an α-actinin–AcGFP-expressing myocyte in a mouse showing the highest HR value in Fig. 5E (i.e., 523 bpm). Bar, 10 µm.
Figure S4. Sarcomeres in a myocyte in a beating heart in vivo with image sequence reconstruction. (A; top) Confocal image of the myocyte at phase $-8$, which is the same as that in Fig. 6 B, bottom. Bar, 20 µm. (Bottom) Plot profile of the yellow outlined rectangular region in the myocyte at phase $-2$, which is the same as that in Fig. 6 B, bottom. SL, 1.76 ± 0.19 µm. (B; top) Confocal image of the myocyte at phase $-2$, which is the same as that in Fig. 6 B, bottom. Bar, 20 µm. (Bottom) Plot profile of the yellow outlined rectangular region in the myocyte at phase $-2$, which is the same as that in Fig. 6 B, bottom. SL, 1.69 ± 0.12 µm (P > 0.05 compared with the value in A). (C; top) Confocal image of the myocyte at phase 0, which is the same as that in Fig. 6 B, bottom. Bar, 20 µm. (Bottom) Plot profile of the yellow outlined rectangular region in the myocyte at phase 0, which is the same as that in Fig. 6 B, bottom. SL, 1.60 ± 0.08 µm (P < 0.05 compared with the value in A; P > 0.05 compared with the value in B). (D; top) Confocal image of the myocyte at phase 7, which is the same as that in Fig. 6 B, bottom. Bar, 20 µm. (Bottom) Plot profile of the yellow outlined rectangular region in the myocyte at phase 7, which is the same as that in Fig. 6 B, bottom. SL, 1.73 ± 0.06 µm (P < 0.05 compared with the value in C; P > 0.05 compared with the value in A or B). Note that despite differing SL values, the images were in focus throughout the cardiac cycle, which is described in detail in Fig. 6 (compare Fig. 4 B). Length of the myocyte in the image plane, >50 µm throughout the cardiac cycle. Sarcomeres indicated by yellow lines were likewise used to derive the average SL values in Fig. 6 C. (E) Relation of phase (from $-8$ to 8; see Fig. 6) versus F.I. at various Z-directions (from 0 to 7 µm in the downward direction). F.I. obtained from an AcGFP-expressing myocyte (same as that in Fig. 6 B). Data represent average values obtained from 267 cardiac cycles. Pseudo colors used to indicate the level of F.I. Note that the Z-axis position varies as phase changes (~5 µm in the transition from diastole to systole and vice versa). (F) Quantified graph showing maximal F.I. (along the Z-direction) at various phases (analyzed from E). In-focus images have greater numbers than defocused images because of high levels of AcGFP fluorescence. Note that the difference between the maximal and minimal values (shown by arrows) is less than ~10%, showing the stability of F.I., despite the movement of the myocyte (see also A–D).
Figure S5. Cardiac sarcomere imaging with image sequence reconstruction in a mouse showing high systolic LVP. (A) Time-dependent changes in SL (red) and LVP (black) obtained from the reconstructed image sequence (as in Fig. 6 C). SL values were averaged at various phases from the reconstructed image sequence. LVP values were averaged at various phases from the data of 738 cardiac cycles. Error bars represent mean ± SEM. (B) Relationship between SL and LVP during the cardiac cycle. Average values of SL and LVP obtained in A were plotted. Colors indicate the phases in the cardiac cycle (as in right). (C; top) Confocal images of the myocyte at phase −4 (left) and 0 (right). Bar, 20 µm. Sarcomeres indicated by red and yellow outlined rectangular region were used to derive the average SL values in A and B. SL values were 1.66 ± 0.09 and 1.59 ± 0.24 µm at phases −4 and 0, respectively. (Bottom) Plot profile of the red outlined rectangular region in the myocyte in top. Despite differing SL values, the images were in focus throughout the cardiac cycle, at both phase −4 (~0 mmHg) and 0 (~80 mmHg).

Video 1. Ventricular myocyte in the beating heart in vivo (without image sequence reconstruction). Cardiomyocyte expressing α-actinin–AcGFP located in the central part of the LV was imaged (see Fig. 4). Objective lens, 40× (N/A, 0.80; water immersion). Speed, 100 fps. Bar, 10 µm.

Video 2. Ventricular myocyte in the beating heart in vivo with image sequence reconstruction. Cardiomyocyte expressing α-actinin–AcGFP located in the central part of the LV was imaged (see Figs. 6B and S4, A–D). Objective lens, 60× (N/A, 1.00; water immersion). Speed, 100 fps. Bar, 10 µm.

Video 3. Ventricular myocyte in the beating heart in vivo without image sequence reconstruction. Cardiomyocyte is the same as in Video 2. Note periodic defocused images as a result of movement of the heart. Objective lens, 60× (N/A, 1.00; water immersion). Speed, 100 fps. Bar, 10 µm.