

Fast Measurement of Sarcomere Length and Cell Orientation in Langendorff-Perfused Hearts Using Remote Focusing Microscopy

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Rationale: Sarcomere length (SL) is a key indicator of cardiac mechanical function, but current imaging technologies are limited in their ability to unambiguously measure and characterize SL at the cell level in intact, living tissue.

Objective: We developed a method for measuring SL and regional cell orientation using remote focusing microscopy, an emerging imaging modality that can capture light from arbitrary oblique planes within a sample.

Methods and Results: We present a protocol that unambiguously and quickly determines cell orientation from user-selected areas in a field of view by imaging 2 oblique planes that share a common major axis with the cell. We demonstrate the effectiveness of the technique in establishing single-cell SL in Langendorff-perfused hearts loaded with the membrane dye di-4-ANEPPS.

Conclusions: Remote focusing microscopy can measure cell orientation in complex 2-photon data sets without capturing full z stacks. The technique allows rapid assessment of SL in healthy and diseased heart experimental preparations. (*Circ Res.* 2013;113:863-870.)

Key Words: instrumentation ■ multiphoton fluorescence microscopy ■ optical imaging ■ sarcomeres

Optical sectioning microscopes are finding increased use as a tool to monitor structure and function at the cell level in the living Langendorff-perfused heart.^{1,2} Confocal (1-photon) and 2-photon imaging modalities have been used to study calcium³⁻⁶ and voltage⁷⁻¹⁰ transients at cellular and subcellular¹¹ resolutions in the intact organ. Physiologically important parameters, such as sarcomere spacing^{12,13} and intracellular calcium wave front speeds,¹⁴⁻¹⁷ can also be directly measured. Given the anisotropic nature of myocardial tissue organization, quantification of measurements with a spatial component (distance or velocity) assumes that the cell orientation relative to the image plane is known or can be ignored. This assumption, however, is often not valid in anatomically complex tissues. Cell orientation can be identified by capturing images of planes at various depths (z stacks), but z stacks take several minutes to acquire, which can be a rate-limiting step in time-sensitive biological preparations. Furthermore, tissue may be adversely affected by pharmacological or physical immobilization procedures that may be required for accurate image registration during the long acquisition process.

We recently proposed a method for measuring sarcomere length (SL) in near-epicardial layers of Langendorff-perfused

hearts based on conventional 2-photon measurements of di-4-ANEPPS fluorescence.¹³ The technique we proposed captured detailed images of cell structure and allowed for direct quantification of local SL variations. However, there was 1 key caveat: because the angle of the tissue relative to the imaging plane is unknown, each measured SL may be skewed toward larger values. In addition, although hearts were arrested, it proved to be impossible to identify cell orientation from z stacks because of residual tissue motion. The time needed to capture z stacks was also problematic as SL increased as a function of experimental time as a result of progressive tissue edema, which made multisite characterization and comparison difficult. We proposed statistical techniques based on Monte-Carlo simulations to estimate true SL from image data sets. Nonetheless, the accuracy of any individual SL measurement and related measures, such as local SL variability, remained poorly constrained.

Here, we introduce an SL measurement technique based on an emerging imaging modality known as remote focusing microscopy. Conventional confocal and 2-photon microscopes are restricted to imaging quickly in image planes perpendicular to the optic axis (the xy plane) and slowly in axial (z) direction.

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Nonstandard Abbreviations and Acronyms

SL	sarcomere length
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This limitation is the result of the difficulty associated with quickly translating the imaging objective or stage because of their relatively large inertial mass. Conventional microscopes cannot move the focal spot optically in the axial direction because this results in the degradation of both the shape and intensity of the spot, even for modest displacements from the focal plane.¹⁸ Recently, we have shown that by using a different imaging architecture, the focal spot can be translated throughout a 3-dimensional (3D) volume at high speed while still maintaining diffraction-limited performance.¹⁹ The approach was validated by monitoring calcium transients in a population of bulk-loaded neurons from a large volume of brain tissue,²⁰ where aberration-free functional responses from different neurons, separated by >60 μm in depth, were measured with millisecond time resolution. In the present study, we adapted the microscope to unambiguously measure both SL and cell orientation at high speed in intact Langendorff-perfused rat hearts.

Methods

An expanded Methods section is available in the online-only Data Supplement.

Langendorff-Perfused Heart Preparation

The investigation conformed to the Animals (Scientific Procedures) Act 1986 (United Kingdom). Hearts were isolated from female rats (≈ 250 – 300 g) killed by an overdose of anesthetic (pentobarbital), rinsed in ice-cold Tyrode buffer, and mounted to a Langendorff system for coronary perfusion with normal Tyrode. Three strains were used (Sprague-Dawley, Wistar-Kyoto, and spontaneously hypertensive rats), but results are treated as 1 group for the purpose of this report.

Imaging

The following is a description of implementing SL measurements using the arbitrary path microscope described by Botcherby et al.²⁰ Other fast z-scanning microscope implementations are reviewed in the Discussion section.

Remote Focusing Microscopy

We have implemented a novel 2-photon microscope that is capable of high-speed scanning throughout a sample volume without translating the specimen or objective. A complete description is given in the online-only Data Supplement, but the general principle is explained here. The illumination spot in an infinity-corrected microscope can be translated along the z axis by illuminating the objective lens with a nonparallel (convergent or divergent) beam of light. The problem is that unless this beam has a specific wave front profile, severe optical aberrations may give rise to a reduction in image quality. This is particularly a problem when imaging with high numeric aperture objectives, as is often the case in life science applications using fluorescent reporters of structure or function.²¹ To obtain diffraction-limited performance, it is necessary to shape the exact wave front profiles required to refocus. The strategy we used here is use of a second matched objective to produce this wave front, as shown in Figure 1.

The second matched high numeric aperture objective (L2) appeared in the axial scan unit, and remote focusing was achieved by moving the lightweight scanning mirror (M). Compared with previous inertia-limited specimen scanning systems,²² which permit maximum scan speeds of ≈ 20 Hz in the z direction, we have demonstrated that rates ≤ 2.7 kHz are achievable with our system because of the low mass of the scanning mirror. The scan speed in the z direction was comparable with galvanometer scan rates in the xy plane.

Imaging Strategy

A high-resolution (512 $\times$ 512 pixel), full field of view, conventional focal plane (xy) image is first obtained from an area of interest within the cardiac tissue. Using custom-built software (Labview), the user marks 2 points (r_1 and r_2) in the full field image, which define a linear path that is approximately aligned to the apparent principal axis of one of the cells. The software then defines 2 orthogonal planes, which pass through this primary axis and are inclined at $+45^\circ$ and -45° to the xy plane (Figure 2). Trajectories are then calculated to scan the focal spot in a raster fashion to acquire images from these 2 oblique planes one after the other. The width of each image is defined by the user and is usually selected to cover the width of the cell in question. Two planes can be acquired in <200 ms; the exact value ultimately depends on operator-defined size and resolution of the oblique image planes. This process is repeated for different cells at user-defined locations throughout the field of view.

Angle Calculations

The tilt angle of the cell can be calculated by finding the vector in x,y,z space that projects along the long axis of the cell perpendicular to the striation orientation. This $\mathbf{K}$ vector ($\mathbf{K}_{\text{CELL}}$) describes the wave number associated with the sarcomere spacing and can be defined by its projection onto the $+45^\circ$ (x',y') and -45° (x'',y'') orthogonal planes:

$$\mathbf{K}_{\text{CELL}} = \left(\frac{k_{x''} + k_{x'}}{\sqrt{2}} \right) \hat{x} + \left(\frac{k_{y''} + k_{y'}}{2} \right) \hat{y} + \left(\frac{k_{x'} - k_{x''}}{\sqrt{2}} \right) \hat{z} \quad (1)$$

where ($\hat{x}, \hat{y}, \hat{z}$) are unit vectors in the x , y , and z directions, and ($k_{x'}$, $k_{y'}$) and ($k_{x''}$, $k_{y''}$) are the projections of the $\mathbf{K}_{\text{CELL}}$ vector on the $+45^\circ$ and -45° planes, respectively. The projection ($k_{x'}$, $k_{y'}$) can be found directly from the striation spacing and orientation on the $+45^\circ$ plane by projecting the data into frequency (Fourier) space:

$$k_{x'} = \frac{2\pi}{SL_{+45^\circ}} \sin(\theta_{+45^\circ})$$

$$k_{y'} = \frac{2\pi}{SL_{+45^\circ}} \cos(\theta_{+45^\circ})$$

$$k_{x''} = \frac{2\pi}{SL_{-45^\circ}} \sin(\theta_{-45^\circ})$$

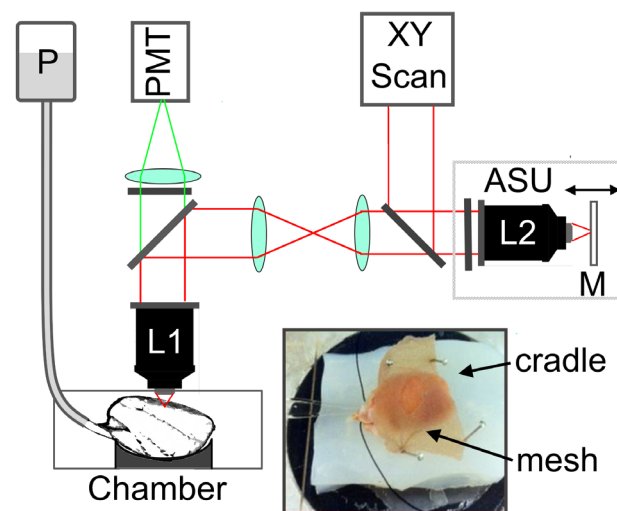


Figure 1. Remote focusing arbitrary path microscopy of the living heart. An image of a perfused heart is shown in the inset. ASU indicates axial scan unit; L1 and L2, imaging lens and reference lens; M, mirror; P, gravity-fed perfusion; PMT, photomultiplier tube.

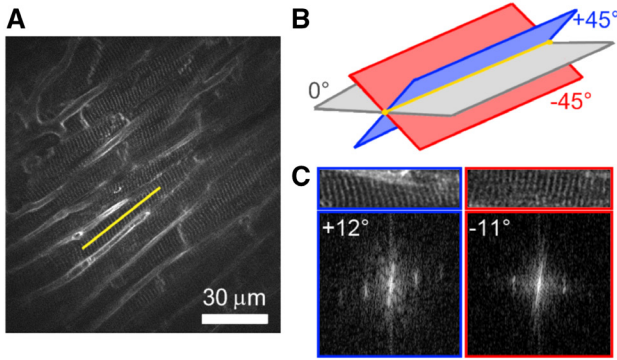


Figure 2. Capture of 2 oblique image planes. **A**, Two-photon image of dye-loaded heart captured at 0° (the conventional *xy* imaging plane). The operator defines a path along the apparent cell axis (yellow line). **B**, Oblique planes, coaxial to the user-defined path in **(A)**, are defined at +45° (blue) and -45° (red) relative to the conventional imaging plane (gray). **C**, Images (top, magnified by a factor of 1.5 relative to **A**) and their Fourier transforms (bottom) for both oblique planes. The Fourier transform allows direct determination of the striation angle in the image by searching for a local maxima, centered at a spatial frequency corresponding to biologically reasonable sarcomere length ranges.

$$k_{y''} = \frac{2\pi}{SL_{-45^\circ}} \cos(\theta_{-45^\circ})$$

where θ_{+45° is the cell's orientation in the +45° plane taken from a line running perpendicular to the striations, and SL_{+45° is the sarcomere length in the +45° plane taken along the same perpendicular line. The values of $(k_{x'}, k_{y'})$ are found by applying the same steps on the striations in the -45° plane image. The cell tilt angle α can then be directly calculated by dividing the z component of $\mathbf{K}_{CELL}$ by the magnitude of the whole vector:

$$\alpha = \sin^{-1} \left(\left[\frac{k_{x'} - k_{y'}}{\sqrt{2}} \right] / K_{CELL} \right) \quad (2)$$

In practice, a simple approximation that does not depend on measuring SL in the oblique planes was used to analyze the data in this study. Tissue tilt angle α can be closely approximated by the following equation:

$$\alpha = \text{atan} \left(\sqrt{2} \tan \frac{\theta_{+45^\circ} - \theta_{-45^\circ}}{2} \right) \quad (3)$$

Details on the use of Equation 3 are given in the online-only Data Supplement.

Resolution Limitations

The maximum angle that can be measured in the oblique plane images is constrained by a reduction in spatial resolution in the axial (z -)direction, a feature that is common to all microscope objectives. Oblique plane images will have a reduced resolution compared with focal plane images.²³ Simulations were performed to determine the maximum resolvable angle, given that sarcomeres are resolvable with 1 μm resolution in the *xy* plane. We found that our method should resolve cell tilt angles, α , up to 30° (Figure VIII in the online-only Data Supplement).

Measurement Validation by Comparison With a Rigid Cell Model

We created a rotatable cell model to validate Equations 1 to 3 and to generate simulated sarcomere profiles to compare with real data. The software allows input of cell rotation around the x , y , and z axes and

outputs images of striations as they would appear in the 0° (*xy* focal plane), as well as in +45° and -45° oblique planes (Figure 3). Data from the oblique planes are considered valid and used for subsequent SL corrections only if all 3 image planes are consistent with the model cell. To determine consistency between the raw image data and the cell model, a comparison is made in the Fourier domain (Figure IV in the online-only Data Supplement).

To obtain the raw image data, the 0° (*xy*) image plane is first extracted as a subset of the full field image, as shown in Figure 3A. The angle of the striation pattern within the 0°, +45°, and -45° image planes (θ_0 , θ_{+45° , and θ_{-45° , respectively) is measured by finding the location of the local maxima in the Fourier-transformed images. To ensure that the correct harmonic of the transform is used to calculate striation inclination angle, the maxima are identified within a band-limited region of the Fourier spectrum that corresponds to the expected range of SL values (Figures IVc and IVd in the online-only Data Supplement). If the spatial frequency maxima are well-localized, an estimate of α can be determined by Equation 2. To validate this estimate, this value of α is inserted into the cell model and an image of the 0° plane is obtained. If the cell model value of the independent variable θ_0 is within 5° of the value obtained from the raw image data, then the estimate of α is accepted.

In some cases, noise or motion in the image results in a Fourier transform with a broad peak, which may be consistent with a range of striation angles. In these cases, the average energy for a 10×10 pixel region, centered on the local intensity maximum, is compared with the average intensity centered on a point predicted by the model cell for each subplane. The estimate of α is accepted if the 10×10 pixel region around the frequency maximum obtained for the cell model image contains >95% of the spectral energy measured for the raw data (Equation IV and Figures II and III in the online-only Data Supplement). Angle estimates are rejected if striations in the 0° plane are not within 5° of the model cell prediction, and the average intensity centered on the peak is not consistent with the model cell prediction for each subplane.

Correcting SL in the Full Field of View Image

The data are processed offline to identify cell orientation and to correct for SL measurement errors in the full-field, focal plane (*xy*) image. First, the operator determines α for the cells measured at oblique angles, as described. The operator then measures SL of cells immediately adjacent to these cells in the original full field of view (512×512 pixel) image using custom-written software (available on request) that finds the peak of the discrete Fourier transform from the long axis of user-selected cells in the image.¹³ The measured SL is converted to the real SL by multiplying each measure by the cosine of the tilt angle α .

Results

Rapid Measurement of Cell Orientation and SL

Figure 4 shows representative results obtained using the remote focusing microscope. A full field of view image, along with 0°, +45°, and -45° subplanes, was captured as detailed in the Methods section. Once focused to the desired depth, a full field of view with 2 pairs of oblique plane images can be captured in <3 minutes.

Regional Variations in Cell Tilt

Offline processing of the oblique and 0° image planes demonstrates that different cell alignment angles may be present within 1 field of view. Figure 4A and 4B shows striation data captured from 2 different subepicardial locations of the same rat ventricle. The composite image on the right of each panel shows the 0°, +45°, and -45° planes (raw column), the Fourier-transformed images (fft column), and the 0°, +45°, and -45° planes extracted from the model cell (model column; rotated

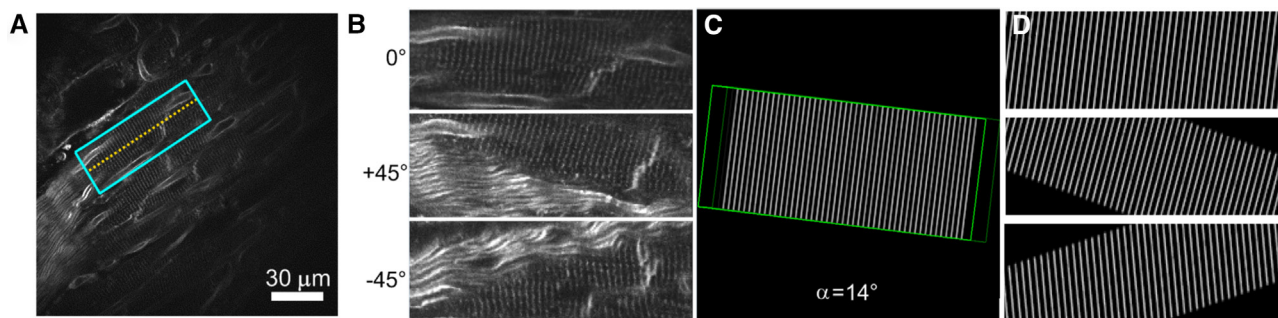


Figure 3. Comparison of imaging data with a model cell. **A**, A subsection of the conventional focal plane (0°) image is extracted for comparison with the oblique plane images. **B**, The $+45^\circ$ and -45° oblique plane images are used to uniquely determine the cell orientation. In this case, $\alpha = 14^\circ$. The panels are magnified by a factor of 2 relative to **(A)**. **C**, A model of the cell using the measured sarcomere length and calculated orientation is created. **D**, The imaging trajectories used in the experiment are applied to the model cell in **(C)** to extract the expected images in the 0° , $+45^\circ$, and -45° imaging planes for direct comparison with the raw data.

to fit the observed data). The overlaid angle values in the fft and model columns show the inclination angle of the striation pattern with respect to the long axis of the image for each of the 3 planes (ie, θ_{0° , θ_{+45° , and θ_{-45°).

For both cases shown in Figure 4A, there is good agreement (a maximum difference of 3°) between the angles found in the 3 image planes and the 3D-simulated cell model, which indicates that the source data are relatively free from artifacts, with high signal-to-noise ratio, accurately representing the true angle of cell alignment within the tissue. The tissue tilt angle α calculated for the 2 user-defined paths gives the same value in Figure 4A (11°). However, for a different region of tissue, shown in Figure 4B, the calculated value of α is different for paths 1 (8°) and 2 (20°). The observed angle differences are reflected in local SL measurements. SLs were measured for 8 cells within the high-resolution image in Figure 4A, giving an uncorrected average SL of $2.23 \pm 0.03 \mu\text{m}$. In contrast, SLs captured from 20 cells in Figure 4B display a higher variance ($2.28 \pm 0.08 \mu\text{m}$). The variance observed is consistent with the angles measured at the 2 locations. The cell defined by the first (yellow) path in Figure 4B has a markedly lower SL ($2.23 \mu\text{m}$) than the one defined by the second (blue) path ($2.33 \mu\text{m}$). In this case, the differences in apparent

SL (in the xy plane) can be accounted for by the calculated cell tilt angle. The corrected SLs of the cells, identified by paths 1 and 2, are 2.21 and $2.19 \mu\text{m}$, respectively. Although we do not have angle data for the majority of the cells contained in the high-resolution images, SL measurements suggest that cells in the tissue may be oriented on 2 distinct planes in Figure 4B, which is consistent with recent data on transmural divergence of myolaminar structures (sheets) from the plane tangential to the epicardium.²⁴ The blue plus symbols indicate cells with SL greater than the average, and the yellow minus symbols indicate cells with SL lower than the average; the spatial distribution of SL measurements is clustered, with longer SLs observed primarily near path 2. We also note that the relatively low contrast region between the 2 user-defined paths provides a visual cue that the high-resolution image may contain cells organized in 2 intersecting tissue sheets.

Angle Corrections Change Distribution.

The distribution of SLs extracted from 1 experimental preparation is shown in Figure 5. Here, we collected SLs from cells positioned along or immediately adjacent to user-defined paths. Additional SL measurements were made in images where analyses of the oblique plane images suggest a consistent cell tilt angle across the field of view. The histogram

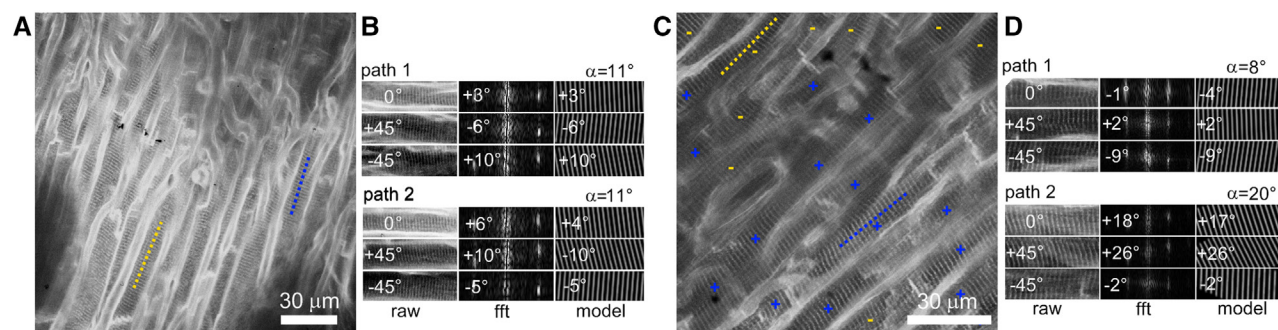


Figure 4. Variability in cell tilt angle α across the field of view. **A**, An example of a tissue region where α is uniform across the field of view. Two user-defined paths (yellow and blue dashed lines) are highlighted within a 512×512 resolution conventional focal plane image. **B**, Raw and processed data for path 1 (yellow dashed path in **A**) and path 2 (blue dashed path in **A**). The columns of the image matrix for paths 1 and 2 show the raw images, Fourier-transformed raw images, and the computer model reconstruction of the image plane data, respectively. The rows of the image matrix correspond to image data taken from the conventional focal plane (0°) and the 2 oblique image planes ($+45^\circ$ and -45°). Both paths produce an estimate of $\alpha = 11^\circ$. **C**, An example of a tissue region where α shows 2 distinct angles (8° and 20°) in 1 field of view. The blue + symbols indicate cells with sarcomere length (SL) greater than the average, and the yellow - symbols indicate cells with SL lower than the average. **D**, Raw and processed data for path 1 (yellow dashed path in **C**) and path 2 (blue dashed path in **C**).

for the corrected data set shows, as expected, a shift toward shorter SLs, with the mode changing from 1.80 to 1.75 μm . The SD for SLs decreases from 0.071 to 0.055 μm after correction, a 23% reduction. We note, however, that the data set analyzed in Figure 5 is atypical because it displayed a relatively wide range of cell tilt angles ($8 \pm 12^\circ$). We observed more modest reductions in variance in the majority (6/8) of the analyzed data sets, with an average reduction in SL SD of 17%. The average corrected SL during 8 experiments is $2.09 \pm 0.168 \mu\text{m}$.

Angle Distribution

Our methods captured a wide range of cell tilt angles, ranging from 0° to 26° . The distribution of measured angles is not random (Figure 6, bottom), and we find that steep tissue angles are relatively rare in most of our data sets. We do, however, observe a clear correlation between measured (uncorrected) SL and tissue angle. The relationship between calculated angle and SL is shown in Figure 6 (top) for all cells for which the tilt angle α was measured in all 8 experiments. In contrast to the analysis shown in Figure 5 for a single heart, here we plotted only SLs collected from cells running along or immediately adjacent to user-defined paths and excluded measurements from all other cells in the high-resolution image. To better visualize the change in SL and compensate for interpreparation differences, we plotted the change in SL (ΔSL) as a function of measured cell angle. The average-corrected SL for each data set was subtracted from every measured SL to give a ΔSL value in that data set, and ΔSL values were plotted for all 8 experimental runs. ΔSL values showed an overall upward trend as a function of cell tilt. We fit the curve $\Delta\text{SL}(\alpha) = \text{SL}_f \cos(\alpha)^{-1} - \text{SL}_p$ with SL_f as the free variable using a nonlinear least-squares Marquardt-Levenberg algorithm (Gnuplot), and the result showed good agreement with the data, yielding an $\text{SL}_f = 1.91 \pm 0.11 \mu\text{m}$ (Figure 6A, dashed line). Additional fits using filtered data sets are shown in Figure IV and Table I in the online-only Data Supplement.

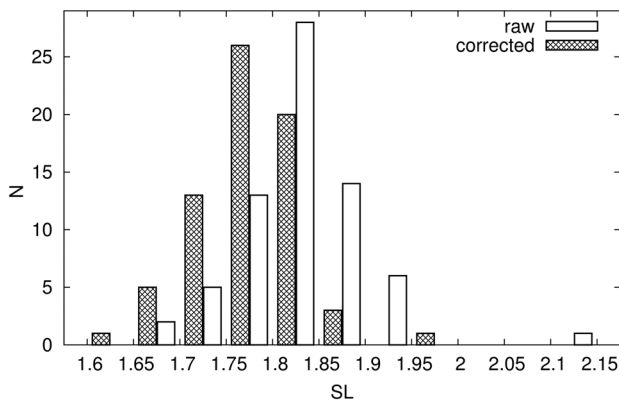


Figure 5. Histogram of sarcomere length (SL) measurements from a single heart preparation. N corresponds to the number of individual cell measurements. SL measurements made directly from the conventional focal plane image and without correction for cell tilt (α) are shown using open bars. SL data that have been corrected using measured values of α are shown using shaded bars. By correcting for cell tilt, the distribution of SL measurements has both shifted the mode of the distribution to shorter SL values (from 1.80 to 1.75 μm) and reduced the SD by 23%.

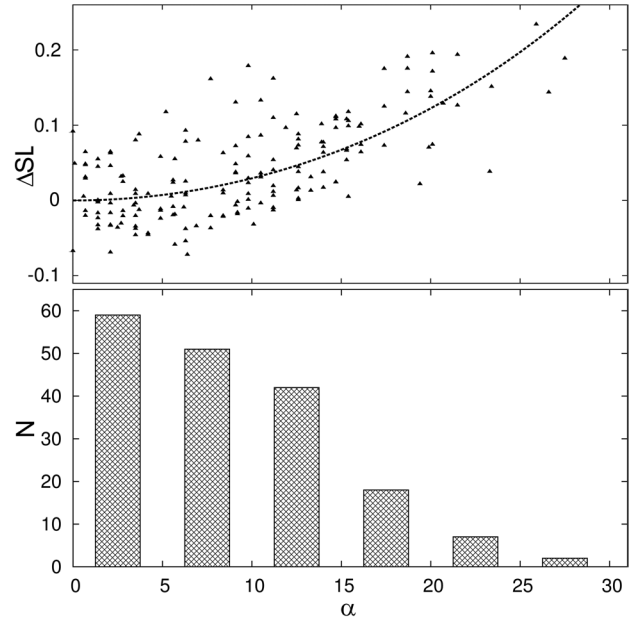


Figure 6. Measured values of α calculated from image data of 178 paths recorded in up to 6 locations across 8 heart preparations. Top, Change in sarcomere length (SL) as a function of cell tilt angle. Change in SL (ΔSL) is obtained for each heart preparation by first calculating the mean α -corrected SL across all measurements. This value is then subtracted from the uncorrected SL measure and correlated against α . The ΔSL values are more robust to variations between heart preparations and allow trends to be identified using all cell measurements. The dashed line shows the line of best fit (SL_f) for the equation $\text{SL}_f / \cos(\alpha) - \text{SL}_p$ with SL_f as the free parameter ($\text{SL}_p = 1.91$). Bottom, Histogram of α values across all measurements.

Discussion

Since its recent introduction,^{19,25} remote focusing microscopy systems have been implemented by a growing number of biophotonics groups interested in imaging fast neuronal dynamics.^{22,26} Here, we present the first use of a remote focusing microscope for cardiac mechanobiology research.

By taking advantage of the system's ability to capture oblique planes at user-defined locations, we demonstrate a new imaging modality capable of rapid capture of myocyte orientation and SL in the intact ex vivo heart. Structural and orientation information can be captured in <3 minutes for any given field of view, which compares favorably with conventional confocal image stack collection, which, for an equivalent task, can take up to 15 minutes (depending on the number of images in the stack). Furthermore, single oblique image plane pairs can be obtained within 200 ms, suitable for time-series studies to explore cell-level responses to an intervention. Because Langendorff-perfused cardiac preparations are subject to significant edema and degradation over time,¹³ there are obvious advantages to obtaining records as fast as possible.

The remote focusing architecture used here is well-suited for capturing sarcomeric structures throughout large tissue volumes. An alternative approach would be to use acousto-optic deflectors to position the focal spot,^{25,27} which allows rapid measurements at arbitrary points in a volume without traversing through an intermediate path. This approach could potentially offer a further significant speed advantage over the

system used in this study in certain applications (eg, when measuring activity from several individual cells dispersed over a large volume); however, acousto-optic deflectors introduce dispersion that impedes diffraction-limited performance from a pulsed laser. In contrast, the galvanometer-based remote focusing system used here is ideal for imaging diffraction-limited contiguous paths and planes, which is desirable when measuring SL over as much of the field of view as possible.

Other microscope architectures are capable of imaging oblique planes and potentially can be used to measure myocyte orientation and SL at high speed. Single-plane illumination microscopy^{28,29} uses a second objective, usually placed at right angles to the imaging objective, to illuminate a plane instead of a spot. Microscopes used in single-plane illumination microscopy can be modified to illuminate samples at oblique angles. However, the use of 2 objectives is only practical with relatively small samples that can be aligned in the focal plane of both objectives. Dunsby et al^{30–32} have developed an oblique plane microscope that uses 1 objective that can illuminate tissue at oblique angles (eg, 60°). Although the oblique plane microscope architecture sacrifices numeric aperture, it may be able to resolve sarcomeric structures. However, the present oblique plane microscope architecture would have to be modified to allow for rapid sequential capture of planes at different (eg, 0° and 45°) angles to allow identification of cell orientation relative to the imaging plane.

Additional steps can be taken to reduce the effects of tissue motion in the preparation. We compared images with a geometric model cell as an exclusion criterion (Figure II in the online-only Data Supplement). However, this process is based on the assumption that sarcomeres are arranged as equally spaced planes throughout a cell, which might not be warranted because sarcomeres are dynamic structures subject to continuous deformation and remodeling,^{33–35} and individual cells can display a range of different SLs.³⁶ The use of the geometric cell model could be avoided if tissue motion is minimized or accounted for. There are several published methods for compensating for tissue motion in whole-organ preparations,^{37–41} which we intend to evaluate and potentially implement in future studies.

In a previous communication,¹³ using data sets collected with a conventional 2-photon microscope, we concluded that SL variance was at least partly attributable to distortions caused by the unknown orientation of the main cell axis relative to the image plane, and we confirm this here (Figures 4–6). Any cell tilt (α) necessarily results in an increase in the apparent SL seen in the *xy* plane, and variability in α will widen and skew the frequency distribution of SLs to longer values. The Monte-Carlo simulations in our earlier work led us to suggest that measurement of the distribution mode gives an accurate indicator of the median SL in conventionally acquired data sets, given a large sample size and the assumption of randomly distributed cell tilt angles. In the present study, however, we observed 1 case (Figure 5) in which the mode of the distribution shifted after correcting for tissue angle. This discrepancy may be because of the relatively small size of the data set in Figure 5 but may also be caused by a relatively large number of different and steep tissue angles found in that particular data set.

The absolute value of diastolic SL reported here ($2.09 \pm 0.168 \mu\text{m}$) is consistent with results from other groups ($1.96\text{--}2.1 \mu\text{m}$ for histological⁴² and x-ray diffraction studies,⁴³ respectively). However, it is higher than the value we previously obtained in a similar preparation ($1.95 \mu\text{m}$)¹³ using a conventional 2-photon microscope. The most probable explanation for this difference is tissue edema. As we reported previously, edema dramatically increases muscle volume and SL as a function of time since tissue isolation.^{13,44} This played a larger role here because of logistical constraints that increased the time to first measurements by ≈ 10 minutes, compared with our previous report (see online-only Data Supplement [Absolute SL]). Experiments were also performed with different perfusion solutions and different rat strains, which may have impacted SL and resulted in large interexperiment variability. Notably, we used the motion-uncoupler blebbistatin in some experiments, which is known to increase diastolic SL.⁴⁵ Quantifying the differences in absolute SL as a function of perfusion solution and phenotype is the subject of ongoing experiments. However, the goal of the present study was to validate the remote focusing imaging protocol in a cardiac preparation and to validate the prediction that the measured SL is a function of α . We consider our results (Figures 5 and 6) to be confirmatory in this context.

The measured diastolic SL in the intact Langendorff-perfused heart in this and other studies is larger than those measured in enzymatically isolated single cells (reported values vary between laboratories but are within a range $1.71 \pm 0.10 \mu\text{m}$ ¹³ to $1.93 \pm 0.10 \mu\text{m}$ ⁴⁶). There are 2 environmental factors that might account for these differences. Isolation of single cells or myocardial fragments for ex vivo investigations may alter the complex interactions of cells with their micro-architectural environment,⁴⁷ removing passive strain from the cells, which—if sufficient to affect cell length—would reduce SL. In this case, isolated cell SLs will be shorter than those in situ. In contrast, tissue extraction from the intact animal results in a large increase in tissue volume due to a combination of a release of pressure from the pericardium and tissue edema that is mirrored by a corresponding increase in SL. Diastolic SL in Langendorff-perfused hearts will, therefore, be greater than SLs in the intact animal. A direct comparison of SLs from these ex vivo model systems with those found in the in situ heart will require further development of minimally invasive imaging modalities (eg, microendoscopy)⁴⁸ or the use of blood-perfused preparations,⁴⁴ although both approaches are outside the scope of the present article. However, isolated cells and Langendorff-perfused heart preparations are widely used model systems that are used to inform high-resolution computational models of cardiac structure and function,^{49,50} and quantifying differences between these systems remains an important challenge.

Our new methodology can be directly applied to disease model systems with altered diastolic SL,^{51,52} which at present depend on isolated single-cell data. The technique can also be applied to measurements of SL and its variance at different tissue depths and during variation of ventricular filling pressures. This will facilitate translation of single-cell experiments to the intact heart and, in turn, can be used to obtain SL information to drive subsequent single-cell workloop

studies.⁵³ Further refinements of the technique, in conjunction with emerging methods for tissue immobilization, registration, and tracking,^{37–39} hold the promise of allowing SL measurements in the unarrested beating heart. Experimental measurement of SL during the full cardiac cycle could be used further to refine biophysically accurate electromechanic simulations, which presently rely on a combination of MRI and histological data sets.^{24,50}

The primary focus of this study was to introduce a novel approach toward cell axis and SL measurements. However, we think that our method can be directly applied to any cardiac study in which cellular and subcellular structures play a role in the interpretation of tissue-level data. Studies that use 2-photon techniques to measure action potential characteristics as a function of tissue depth,¹⁰ for example, would immediately benefit from the ability of remote focusing microscope to simultaneously capture structural and functional data while rapidly moving between tissue layers. Rapid capture of cell orientation is especially relevant when considering measurements of calcium wave speed at the cell level in the dye-loaded intact heart, which is an increasingly popular experimental model.^{14–17} Similar techniques may be applicable to diverse biological preparations that use optical sectioning microscopy to measure velocities.^{54,55} We think that remote focusing microscopy techniques have the potential to lead to a better understanding of how function and structure are linked in the intact organ.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Although conventional 2-photon microscopy captures images of myocytes deep within the isolated heart, sarcomere length (SL) cannot be measured directly because the angle of the cell relative to the image plane is unknown.
- Unambiguous measurement of SL in the intact heart normally requires reconstructing a 3-dimensional (3D) representation of the cell from a series of images captured at different depths (a z stack).
- In addition to being time-consuming, conventional z-stack capture requires complete pharmacological immobilization of the heart, limiting its applicability.

What New Information Does This Article Contribute?

- We used a newly developed imaging modality called remote focusing microscopy to quickly capture 2 subimages of a cell in the intact heart at oblique angles to the image plane and used these images to unambiguously find the SL of the cell.
- Our measurements showed a decrease in mean diastolic SL, as well as a narrowing of the distribution of SLs, compared with those obtained from single, conventionally acquired 2-photon images.

SL is a key indicator of cardiac mechanical function; however, techniques for measuring SL in living, intact tissue are limited. We present a new method based on an emerging imaging modality called remote focusing microscopy that can capture images at oblique angles to the conventional image plane. We showed that the orientation of the cell in 3D space, and by extension the SL of the cell, can be unambiguously and rapidly determined by capturing 2 oblique images along the long axis of the cell. Our method resulted in a reduction in measured absolute SL, as well as its variance, compared with SLs measured from conventionally acquired 2-photon images from the arrested Langendorff-perfused heart preparation. The ability to rapidly measure cell orientation in intact living tissue also has applications to the interpretation of functional parameters, such as calcium wave conduction velocity and morphology. Remote focusing microscopy will facilitate investigations of structure and function at the cell level in the intact, living heart in both healthy and disease model systems.

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Fast Measurement of Sarcomere Length and Cell Orientation in Langendorff-Perfused Hearts Using Remote Focusing Microscopy

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SUPPLEMENTAL MATERIAL

Detailed Methods:

Experimental preparation: Female rats (Sprague-Dawley, Wistar-Kyoto, or Spontaneously Hypertensive; 250-300 g) were rendered unconscious using general anesthesia (3% isoflurane and 97% oxygen), then humanely killed by an approved UK Home Office schedule 1 method according to the Animals (Scientific Procedures) Act of 1986: overdose of pentobarbital followed by dislocation of the neck. The heart was swiftly removed and placed in ice-cold normal Tyrode buffer [containing, in mM: 140 NaCl, 5.4 KCl, 1 MgSO₄, 1.8 CaCl₂, 5 HEPES, 11 Glucose, pH 7.4], for transport to the imaging facility. Hearts were mounted on a gravity-fed Langendorff system and perfused with warm (35-37°C), normal Tyrode for 10 min to resume normal contractions, and loaded with membrane dye di-4-ANEPPS (1-(3-sulfonatopropyl)-4-{beta-[2-(di-N-butylamino)-6-naphthyl]vinyl}pyridinium betaine; Invitrogen), by coronary perfusion (5 μ M over 5 minutes). The perfused heart was then transferred to a custom-made chamber with a resting cradle (Online Figure I), stabilized with nylon mesh, and imaged during gravity perfusion with an approximate flow rate of 5 mL/min, using calcium-free Tyrode or high potassium solution [Tyrode buffer containing 20 mM of KCl]. In some experiments, the motion uncoupler blebbistatin (Sigma-Aldrich) was added at a concentration of 10 μ mol/L.

Microscopic imaging: Imaging was conducted using a custom-built set-up, based around an Olympus BX60M upright microscope, adapted using standard Linos microbench components. The laser source was a Ti:Sapphire laser (Tsunami, Spectra Physics) producing 100 fs pulses centred at 850 nm. An Olympus UAPO W340 40x 1.15NA lens was illuminated at the back aperture with a 90 mW beam to maximise image contrast. After passing through a dichroic mirror (Semrock FF568-Di01-25x36) the two-photon epi-fluorescence was filtered again with a 550-650 nm bandpass filter before being captured with a Hamamatsu H7422P series photon counting PMT.

Laser scanning was achieved with a pair of orthogonally mounted galvanometer mirrors (VM1000+, Cambridge Technology). Synchronous point scanning and data collection were orchestrated through LABVIEW software, running on a reconfigurable FPGA card (NI PCI-7830R, both National Instruments).

Since the galvanometer response is frequency-dependent, the drive signals have to be pre-compensated to ensure the focal spot follows the trajectory accurately. To do this, optical feedback signals from the galvanometers were recorded during trial scans and used to compute phase and amplitude corrections for the driving waveforms. Following compensation, true trajectories are recorded for the purpose of post-processing and are generally found to match desired trajectory to an accuracy of within less than 0.5 μ m.

Estimating cell tilt (α): Application of equations 1 and 2 requires measurement of the apparent sarcomere length (SL), and SL striation tilt in both oblique planes. However, making two additional measurements per cell complicates the measurement protocol and may add additional operator error.

It is straight forward to show that the cell angle α is given by:

Online Equation I.
$$\alpha = \text{atan}(\sqrt{2} \tan(\sigma))$$

where σ can be substituted by θ_{+45} or θ_{-45} . Online equation I is a special case of equations 1 and 2, where there is zero rotation of the cell around the z axis, relative to the intersection of the oblique plane and the image plane. Since the user selects the trajectory to be roughly aligned with the cell's long axis in the x,y imaging plane, this condition is often met in the collected data. Although only a single angled plane is required to determine cell angle, two collected oblique images allow for conditions where one plane does not intersect the cell body sufficiently to give a clear striation pattern in the image. Online Equation I also depends only on one measured variable, instead of four in equations 1 and 2, which reduces the complexity of the analysis. However, if the user-defined path through the cell is significantly ($>5^\circ$) off axis, Online Equation I is not valid. We find that a useful approximation is given by averaging the angles in the two oblique planes (Equation 3, main text).

A Monte Carlo simulation using a geometric model cell shows that Equation 3 gives an acceptable (within 3°) approximation for α compared to Equations 1 and 2 in the main text, when SL is not measured in the oblique planes (within the range of angles shown, Online Figure II). Equation 3 was used to find α for the measurements conducted in this study.

Approximating oblique and in-plane sarcomere striation angles: In some cases, the operator may choose to approximate either the extracted 0° plane, or one of the oblique plane striation angles, e.g. if one or more of the sub-planes have poor signal or ambiguous striations due to tissue motion. The 0° sub-plane angle can be approximated by:

Online Equation II.
$$\theta_0 = \frac{\tan(\theta_{+45}) + \tan(\theta_{-45})}{\sqrt{2}}$$

The oblique plane striation angle can be found by first estimating α using a variant of Eq. 3 (here used to find the striation angle in oblique plane θ_{-45}):

Online Equation III.
$$\alpha = \text{atan}(\sqrt{2} \tan(-\theta_{+45} + \theta_0 / \sqrt{2}))$$

The geometric cell model is rotated by α and the striation angle for θ_{-45} is found by identifying the maximum intensity peak of the Fourier transformed plane. The accuracy of Online Equations II and III were confirmed using Monte Carlo simulations (Online Figure III). In practice, a relatively small fraction of measurements required application of Online Equations II or III (20 of 173 measurements).

Finding striation angle from the local intensity maxima of the Fourier transformed data: Striation angles are found by taking a fast Fourier transformation of a user-defined region within a given sub-plane. The software then finds the local intensity maximum near the expected SL. The operator defines a broad region of interest, where the local intensity peak is expected (between the red lines in Online Figures IV and V).

Model fit: The Fourier transforms of the data can yield broad intensity peaks that may be consistent with a range of tissue angles. The model is considered consistent with the data, if the average intensity of a 10x10 pixel area at the predicted point is greater than, or equal to, 0.95 times the average intensity of a 10x10 pixel area at the local data maxima:

Online Equation IV.
$$\sum_{i,j=-5}^{i,j=5} p(x_m + i, y_m + j) \geq 0.95 \times \sum_{i,j=-5}^{i,j=5} p(x_d + i, y_d + j)$$

where x_m, y_m is the point in Fourier space, predicted by the geometric model, and x_d, y_d is the local maxima in Fourier space (see Online Figures IV and V).

Accounting for tissue motion: Several (3 to 5) repeat measurements were taken at each point, allowing for assessment of the effects of tissue motion on the accuracy of our angle approximations. In most cases, the differences between these sequentially captured images are minor, but some calculated α values varied significantly between runs. The relation in Online Equation IV is used to determine which image in the series is most likely to contain data that represents the true alignment of the cell (Online Figure IV). The α corresponding to the best model fit is saved and applied to the correction of SL measurements.

Accounting for noisy or ambiguous data: In some cases, the geometric model cell does not fit the angles captured in sub-planes. If one or more of the sub-planes contain ‘noisy’ data (represented by indistinct intensity peaks in the Fourier transformed images), the operator can estimate the angle using Online Equations I-III and test whether these new angles are consistent with a rotated model cell by applying Online Equation 4 (see Online Figure V). In practice, approximated angles were used infrequently when analysing the data (20 of 173 data points, Online Figure VII).

Fitting data: Changes in measured SL (ΔSL) should vary as a function of α according to the following equation:

Online Equation V
$$\Delta SL = SL_f / \cos(\alpha) - SL_f$$

Online Equation V is fit to experimentally measured ΔSL using the *fit* command in Gnuplot, which used a nonlinear least-squares Marquardt-Levenberg algorithm (see <http://www.gnuplot.info>). If Online Equation V is valid, we would expect the fit for SL to be close to the average corrected SL, over all experiments. The average corrected SL is $2.09 \pm 0.168 \mu m$ (mean $\pm$ SD). Possible reasons why the fit to Online Equation V underestimates SL are given below.

Applying a quality metric to the data: Each calculated SL value is recorded with two Boolean quality metrics: one to signify that all measured striation angles are within 5° of the rotated model cell, and one to check that all model angles satisfy Online Equation IV. We find that the effects of filtering the data to include only those data points that satisfy both criteria gives relatively minor improvements to the quality of fit, compared to unfiltered data (Online Figure VII).

Measurement error: Any over-estimate of α will shift the corrected SL to lower values. The effects of measurement errors can be seen in Online Figure VI, where the corrected histogram of ΔSL (cross-hatched bars) is skewed to the left. Measurement error may also be responsible for the underestimate for the fit of Online Equation V relative to mean SL in Figure 6 and Online Figure VII. We performed a set of ten simulations, where white noise was added to the angle measurement. For each run, a random number evenly distributed between -2.5 and +2.5 degrees was added to each value of α in the

data set, and the Gnuplot *fit* function was used to determine the best fit for SL in Online Equation V. Adding noise to α lowers the SL_f by an average of $0.075\ \mu\text{m}$ (10 trials, $SD=0.074\ \mu\text{m}$). This value is in relatively close agreement to the difference between the fit to the filtered SL ($2.01\ \mu\text{m}$, Online Table I) and the average corrected SL over 8 experiments ($2.09\pm0.168\ \mu\text{m}$).

Although angle correction results in a decrease in the standard deviation of SL measurements (Online Figure V, SD uncorrected data = 0.063 , SD corrected data = 0.045 for ΔSL), measuring cell angles and applying corrections when the actual tissue is within a few degrees of the image plane (where corrections are unnecessary) will result in an underestimation of SL and potentially increase in the variance of the measurements.

Absolute SL: In a previous study³ we reported measurements of diastolic SL in the Langendorff-perfused heart preparation. The absolute value of diastolic SL in the present study ($2.09\ \mu\text{m}$) is greater than what we previously reported ($1.95\ \mu\text{m}$). This may be caused by the combination of different animal strains (SD, WKY, and SHR) and/or differences in perfusion solutions (high potassium, calcium free, with and without blebbistatin). More probably, it is a result of progressive tissue oedema during crystalloid perfusion, which is a major contributor to SL in Langendorff-perfused hearts. In our earlier study we reported that SL increases as a function of time of exposure to crystalloid solutions (10% between 30 and 75 minutes post sacrifice), and that this correlated with tissue oedema. To mitigate the effects of oedema in the earlier study, we calculated the representative diastolic SL of $1.95\ \mu\text{m}$, based on measurements taken as soon as possible after isolation and within as short a time-window as possible (between 30 and 35 minutes post-sacrifice). In the present study, logistical constraints necessitated the transport of the tissue to a dedicated imaging facility in a different building, which resulted in an additional 10-minute delay between excision of the heart and initial measurements. The diastolic SL value reported here are fully consistent with values obtained previously at time-points corresponding to this delay (greater than 40 minutes post sacrifice in figure 6A in reference 3), as well as with results from other reports ($1.96\ \mu\text{m}$ to $2.1\ \mu\text{m}$ for histological⁴ and x-ray diffraction⁵ studies, respectively).

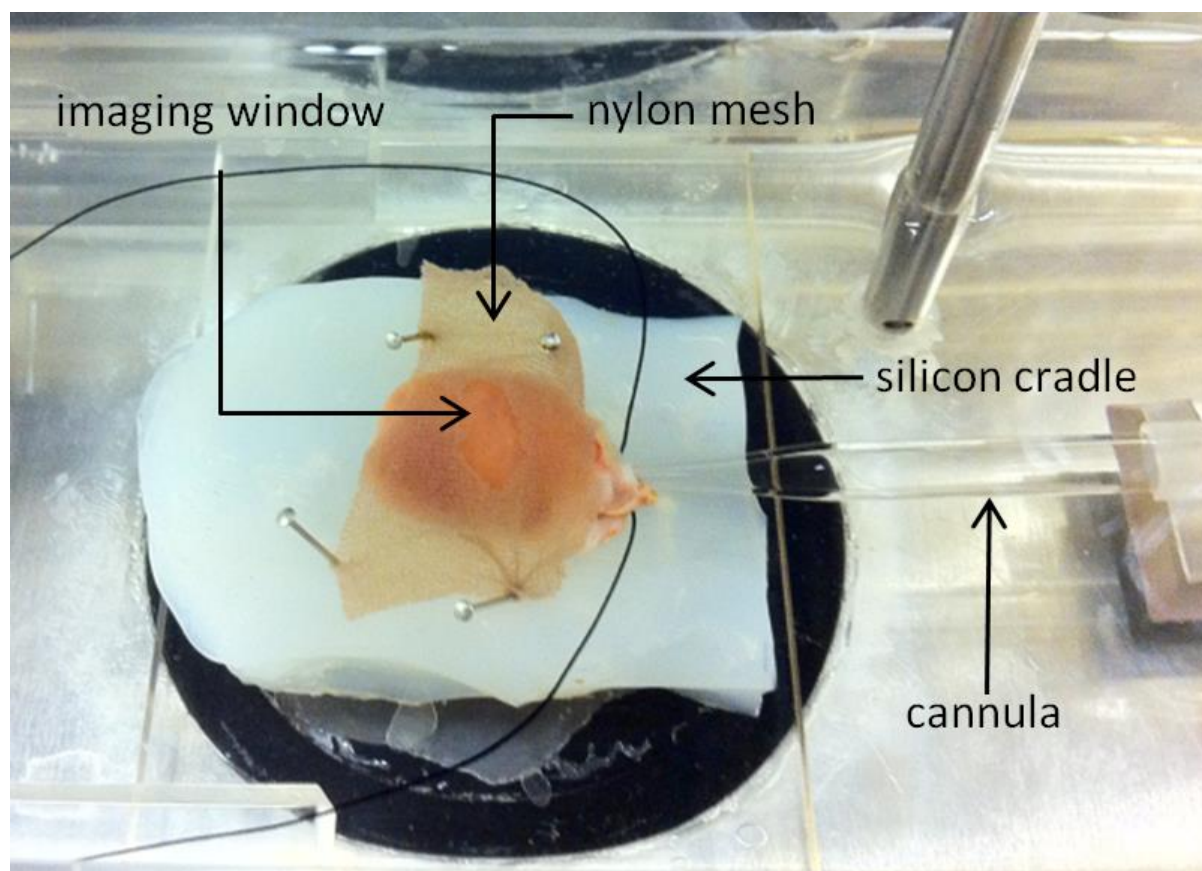
Absolute versus ΔSL : The aim of the present study is validation of a new experimental method for measuring cell tilt, which impacts SL and its variance via geometric effects only, and does not depend on obtaining a physiologically representative measure of (*in situ*-like) diastolic SL. We therefore predominantly report results in terms of ΔSL , which is a measure of the change in SL as a function cell tilt (Figure 6, Online Figures VI and VII). ΔSL is obtained by subtracting the average corrected SL value from each measurement. ΔSL is therefore largely independent of absolute SL, but strongly depends on tissue tilt angle (α). For example, a cell with sarcomere spacing of $1.9\ \mu\text{m}$ will appear as $2.1\ \mu\text{m}$ if the cell has a tilt angle relative to the conventional x,y image plane of 25° ($\Delta SL=0.2\ \mu\text{m}$), however, two cells with the same tilt angle but markedly different SLs of $1.9\ \mu\text{m}$ and $2.1\ \mu\text{m}$ would show a difference in ΔSL of just $0.02\ \mu\text{m}$. The use of ΔSL as a measure to characterise the functionality of our method is also better-suited when combining data sets from different experimental preparations, or where solutions and/or animal strains have differed.

Resolution limitations: The majority of cell tilt angles encountered in this study were small ($<10^\circ$) and none exceeded 26° . In determining the maximum angle that can be addressed using this technique, the main limitation comes from the reduced spatial resolution of oblique plane images, an issue that has been addressed previously by Smith *et al.*² As even high-NA microscope objectives have a limited acceptance angle, the point spread function (PSF) is significantly extended in the axial direction. This results in the axial (z) direction being less well- resolved compared to the x/y-plane. In the imaging

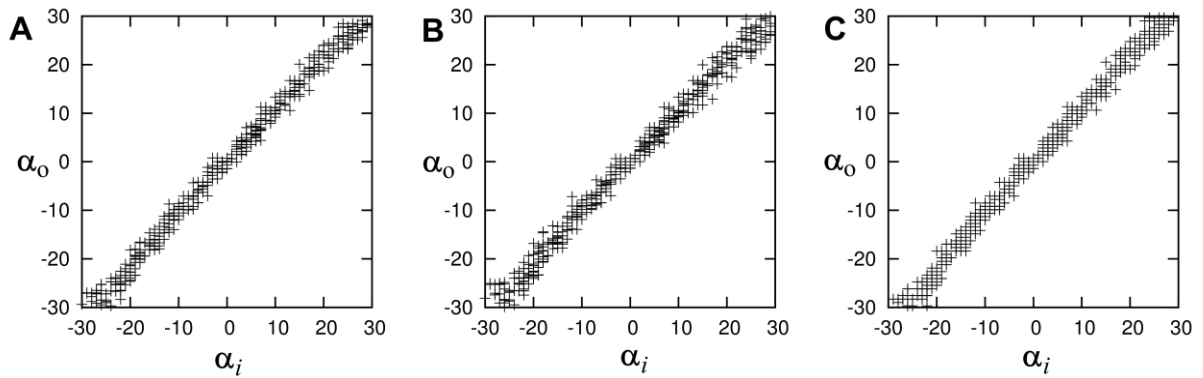
process, the oblique plane object information is convolved with an elliptical PSF, such that the long axis of the ellipse is parallel to the short axis of the oblique plane image. This allows the high frequency sarcomere pattern running parallel to the long edge of the image plane to be sampled at much finer resolution. However, as cell tilt increases, the spatial frequency components along the x' and x'' axes also increase, until the point at which they become unresolvable by the extended PSF, and measurement of the cell tilt is no longer possible. This effect can also be viewed in the spatial frequency plane. The Fourier Transform of the PSF describes the optical transfer function (OTF) of the imaging system. The OTF determines how spatial frequencies in the object plane are mapped to the image plane. Any spatial frequency vector, such as the one describing the sarcomere spacing that falls outside of this OTF will not be resolved by the microscope.

To determine the OTF limit within the oblique planes, a simulation was performed. The simulation took a projection of the cell model in the oblique plane and convolved this with the elliptical point spread function. After the addition of random noise (20% of signal level) the convolved images were then processed in the same way as for the raw imaging data. The recovered estimate for the tilt angle, α , could then be correlated against the value in the model. To obtain a lower bound on α , imposed by the OTF, it was first assumed that the sarcomeres are barely resolvable, having a point spread function size of $1\text{ }\mu\text{m}$ in x,y and $4\text{ }\mu\text{m}$ in z directions. Within the oblique plane this PSF then has projected dimensions of $1\text{ }\mu\text{m} \times 3\text{ }\mu\text{m}$. It can be seen from Online Figure VIII(A) that for this PSF size the maximum extent of the linear region of the measurement is between 17° and 18° . Interestingly, the relationship does become linear periodically after this point, as spatial frequencies are aliased back into the OTF (see reference 2). A more realistic limit imposed by the OTF is provided by inspecting a line transect through the image data. Here it can be seen that feature sizes as small as $1\text{ }\mu\text{m}$ were resolvable by the microscope. The results of re-running the simulations with the PSF size halved in each dimension (doubling the dimensions of the OTF) are shown in Online Figure VIII(B). Here we can see that the oblique angle resolution limits the tilt angle to less than 30° . This is well in line with the maximum angle of 26° identified from the raw data.

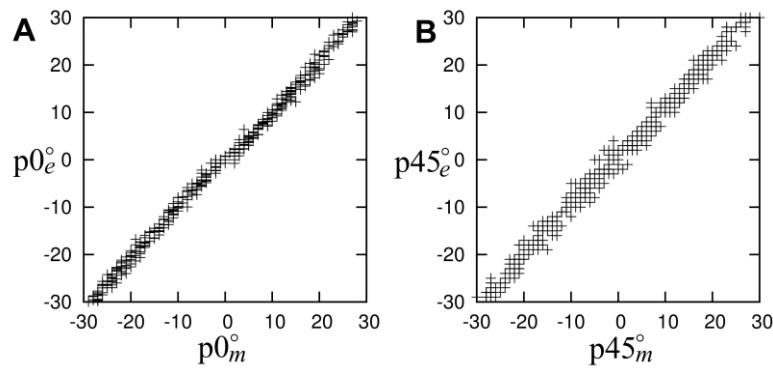
Data supplement Figures:



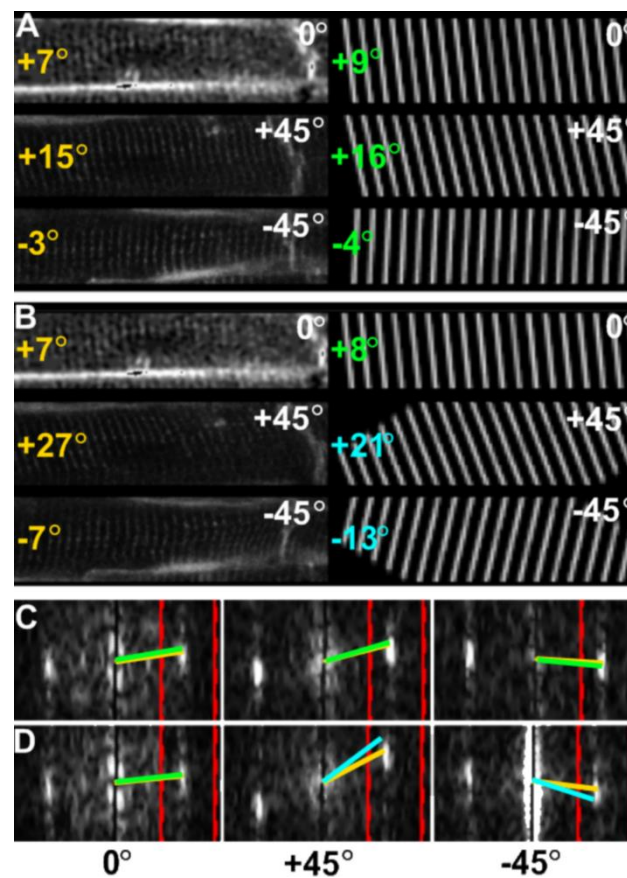
Online Figure I: The Langendorff perfused heart prior to imaging. The heart is restrained using a nylon mesh (commercially available stocking) with a hole cut in the center to allow a clear view of part of the left ventricle. The silicon cradle with a shallow indentation for the heart and cannula is shaped from Sylgard 184 (Dow Corning). The chamber is attached to a metal insert (black circle) that slots into a mechanical stage for alignment.



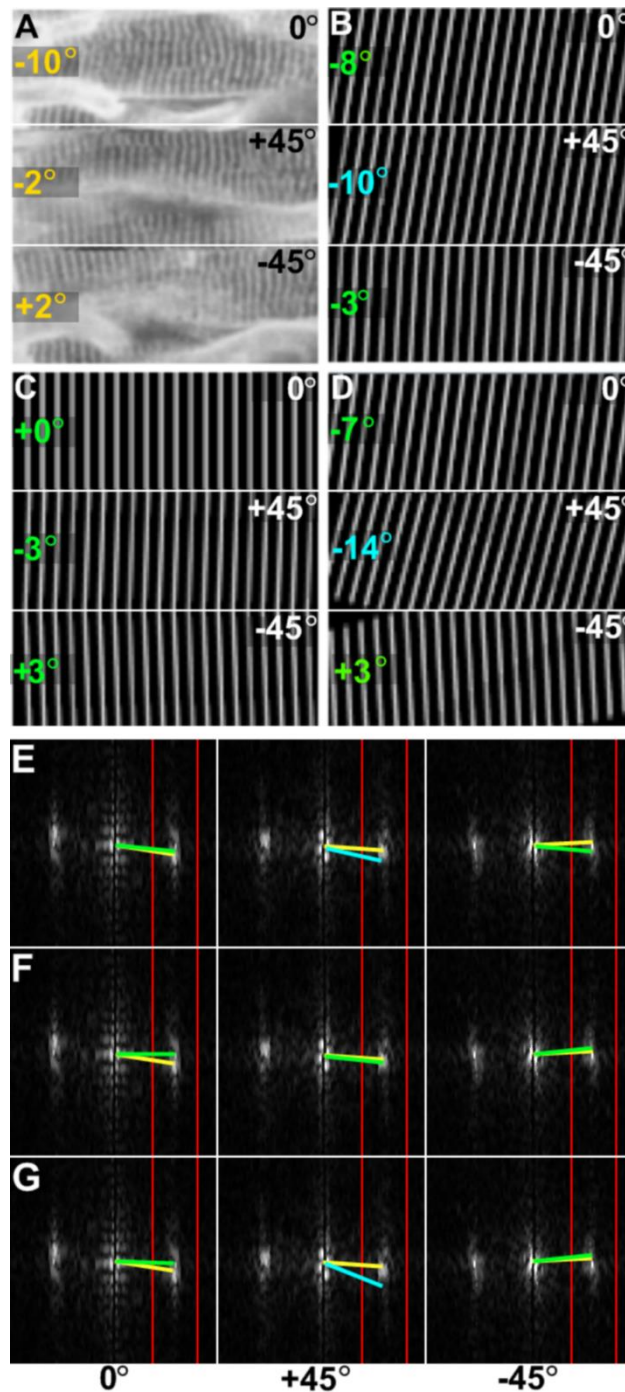
Online Figure II. Model validation. The geometric cell model was used to validate the methodology for finding angles in the experimental data set. For each data point, the model was rotated by applying 3 rotations in the x, y, and z axis from a random distribution within a range of -30° to $+30^\circ$. The graphs show the value of α estimated using Online Equation I (α_0) plotted against the actual value (α_i). (A) The results of applying Equations 1 and 2 to obtain an estimate for α , where SL magnitude is determined from the conventional focal plane image; (B) As for (A) but with SL magnitude set to 1. The mean square error is 0.61° , with a maximum error of 2.5° ; (C) Applying the simplified formula in Online Equation I. The mean square error is 0.37° , with a maximum error of 1.7° .



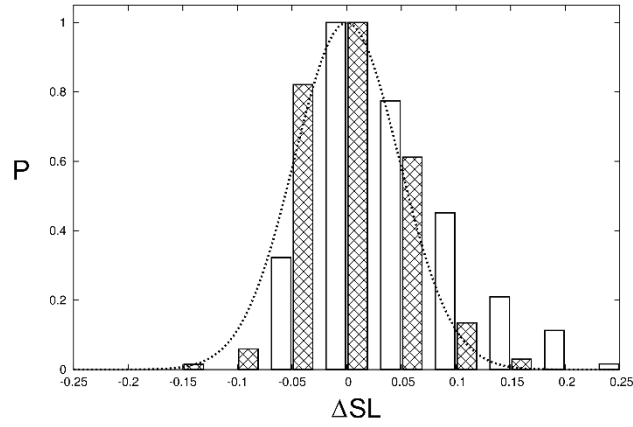
Online Figure III. The plots show the estimated striation angle ($p\theta_e$) plotted against the known striation angle ($p\theta_m$). (A) Estimating the 0° subplane angle using Online Equations II (Mean Square Error=1.9°, maximum error 4.6°); (B) Estimating the +45° subplane by using Online Equation III to find the cells rotation around the y axis, rotating the model by the estimated amount and measuring the model's +45° angle (Mean Square Error 3.7°, maximum error 5°).



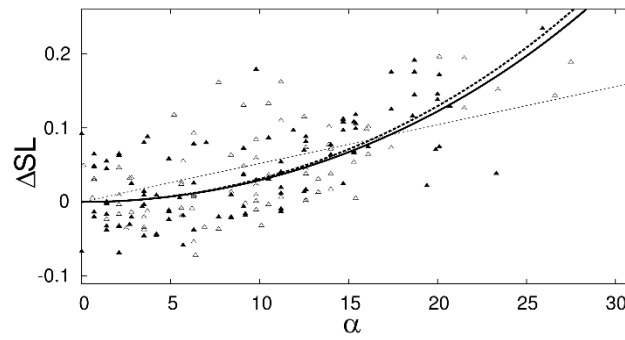
Online Figure IV. The preparation was prone to small contractions during acquisition, which impact angle measurements. (A and B) Two sequentially captured oblique plane measurements (left column) and their model fits (right column). (C and D) The Fourier transforms for the data in panels A and B is shown in panels C and D, with yellow lines drawn from the centre to the local intensity maxima found between the red lines. Green lines show a good model fit, and blue lines show a poor model fit (see Online Equation IV). The two measurements yield two different values of α (14° for A, 24° for B). The measurement shown in A is more consistent with the geometrical model prediction and is saved.



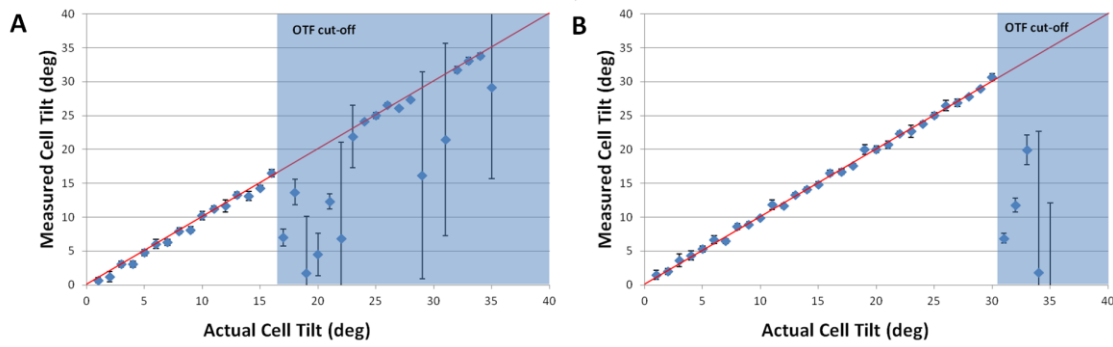
Online Figure V. Choosing the best α from noisy data. A) The three subplanes (0° , $+45^\circ$, -45°). B) The initial model fit based on the data shows that the predicted striation angles in the $+45^\circ$ subplane are not consistent with the data. B) Estimating the angle of the 0° plane using Online Equation I gives a value of 0° . The intensity of the 10×10 pixel region at this point is consistent with the data (Online Equation IV). C) Estimating the angle of the $+45^\circ$ plane using Online Equation II give a predicted value of -14° , but the average intensity of pixels centred at this point is not consistent with the data. The measurement for the 0° fit is considered most accurate and α is set to 4° . E-G) Fourier transforms of the three sub-planes with yellow lines drawn from the centre to the local maxima and green (good fit) or blue (poor fit) lines showing the model fit for panels B-D.



Online Figure VI: Aggregate data over 8 experiments. For each experiment, the average corrected SL (obtained from the average of each measured SL multiplied by $\cos(\alpha)$) is calculated and this value is subtracted from each measured SL (SL_m) and each corrected SL (SL_c) to give ΔSL_m and ΔSL_c . The histograms for all ΔSL_m and ΔSL_c over 8 experiments is plotted (ΔSL_m open bars, ΔSL_c hatched bars). The standard deviation for all $\Delta SL_m = 0.063$, while the standard deviation for all ΔSL_c is 0.045. The dashed line is a Gaussian distribution with standard deviation = 0.045.



Online Figure VII: ΔSL_m plotted against α for all experiments. Filled triangles represent a filtered data set where all model fits are within 5° of the measured data and Online Equation IV is satisfied for each sub-plane. Open triangles plot points where one of these conditions is not satisfied. The data is fit to Online Equation V. The solid curve plots the best fit for all data points ($SL=1.91$) and the heavy dashed curve plots the best fit for the filtered data set ($SL=2.01$). A straight line, with slope of 0.0051 is plotted with a thin dashed line. A summary of the fitting parameters and fit statistics is given in Online Table I.



Online Figure VIII: Effect of PSF size on measured inclination angle for PSF dimensions of (A) 1 μm x 1 μm x 4 μm and (B) 0.5 μm x 0.5 μm x 2 μm . Graph shows the relationship between the imposed values of cell tilt angle, α , on a cell model and the angle measured from projections of the cell model in the oblique planes in the presence of noise. Angle α was calculated from the oblique plane data using the same method as for the raw image data. The greyed-out area shows the region in which the measurement becomes inaccurate as the sarcomere structures are no longer resolved by the imaging objective. Error bars in (A) are calculated from repeating the simulation five times for each imposed inclination angle.

Supplemental Tables and Supporting Information:

	Fit parameter	Reduced Chi-square	Asymptotic standard error
Line (all)	0.0051	1.19	6.4%
Line (filtered)	0.0052	1.20	8.3%
SL (all)	1.91	1.09	5.9%
SL (filtered)	2.01	1.07	7.5%

Online Table I. A summary of the fitting statistics for Online Figure V. The fit for the filtered data is closest to the real average SL over 8 experiments (2.13), and a Chi-square statistic closest to unity, but the fit for the unfiltered data set has the smallest asymptotic standard error. Fits for straight lines are given for comparison.

Supplemental References:

- 1) Fedorov V.V., Lozinsky I.T., Sosunov E.A., Anyukhovskiy E.P., Rosen M.R., et al. (2007) Application of blebbistatin as an excitation-contraction uncoupler for electrophysiologic study of rat and rabbit hearts. *Heart Rhythm* 4: 619–626.
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- 3) Bub G, Camelliti P, Bollensdorff C, Stuckey D.J., Picton G, Burton R.A.B., Clarke K., Kohl P. Measurement and analysis of sarcomere length in rat cardiomyocytes in situ and in vitro. *Am J Physiol Heart Circ Physiol.* 2010;298:H1616–H1625.
- 4) Grimm, A. F., Lin, H. L., & Grimm, B. R. (1980). Left ventricular free wall and intraventricular pressure-sarcomere length distributions. *Am J Physiol Heart Circ Physiol.* 239, H101–H107.
- 5) Yagi, N., Shimizu, J., Mohri, S., Araki, J., Nakamura, K., Okuyama, H., Toyota, H., et al. (2004). X-ray diffraction from a left ventricular wall of rat heart. *Biophys J.* 86, 2286–94.