Stimulated Emission Depletion Live-Cell Super-Resolution Imaging Shows Proliferative Remodeling of T-Tubule Membrane Structures After Myocardial Infarction

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Rationale: Transverse tubules (TTs) couple electric surface signals to remote intracellular Ca\(^{2+}\) release units (CRUs). Diffraction-limited imaging studies have proposed loss of TT components as disease mechanism in heart failure (HF).

Objectives: Objectives were to develop quantitative super-resolution strategies for live-cell imaging of TT membranes in intact cardiomyocytes and to show that TT structures are progressively remodeled during HF development, causing early CRU dysfunction.

Methods and Results: Using stimulated emission depletion (STED) microscopy, we characterized individual TTs with nanometric resolution as direct readout of local membrane morphology 4 and 8 weeks after myocardial infarction (4pMI and 8pMI). Both individual and network TT properties were investigated by quantitative image analysis. The mean area of TT cross sections increased progressively from 4pMI to 8pMI. Unexpectedly, intact TT networks showed differential changes. Longitudinal and oblique TTs were significantly increased at 4pMI, whereas transversal components appeared decreased. Expression of TT-associated proteins junctophilin-2 and caveolin-3 was significantly changed, correlating with network component remodeling. Computational modeling of spatial changes in HF through heterogeneous TT reorganization and RyR2 orphaning (5000 of 20 000 CRUs) uncovered a local mechanism of delayed subcellular Ca\(^{2+}\) release and action potential prolongation.

Conclusions: This study introduces STED nanoscopy for live mapping of TT membrane structures. During early HF development, the local TT morphology and associated proteins were significantly altered, leading to differential network remodeling and Ca\(^{2+}\) release dysynchrony. Our data suggest that TT remodeling during HF development involves proliferative membrane changes, early excitation-contraction uncoupling, and network fracturing. (Circ Res. 2012;111:402-414.)

Key Words: Ca\(^{2+}\) sparks ■ excitation-contraction coupling ■ heart failure ■ T-tubule ■ super-resolution imaging ■ calcium signaling

Due to large volumes of muscle cells, excitation-contraction (E-C) coupling depends critically on fast signaling mechanisms to overcome spatial diffusion barriers. Electron microscopy studies have suggested that transverse tubules (TTs) at sarcomeric Z-line striations enable action potential (AP) propagation. Intracellular voltage imaging provided evidence that TTs propagate APs inside cardiomyocytes. AP propagation by TTs is further supported by synchronization of local Ca\(^{2+}\) release signals (sparks) during E-C coupling in adult ventricular cardiomyocytes. As an E-C coupling mechanism, electron microscopy (EM) studies characterized 12-nm-wide nanodomains containing junctional TT segments and terminal sarcoplasmic reticulum (SR) Ca\(^{2+}\) release sites. Ca\(^{2+}\) release unit (CRU) nanodomains were...
for overview of reported TT changes.\textsuperscript{3,10,11} Lederer et al proposed abnormal spatial organization of the TT network as a mechanism of intracellular Ca\textsuperscript{2+} dysynchrony through “orphaning” of ryanodine receptor (RyR2) Ca\textsuperscript{2+} release channels.\textsuperscript{3} Furthermore, TTs contain L-type Ca\textsuperscript{2+} channels (LCCs) in immediate proximity to RyR2 channels, and TT remodeling may disrupt functional colocalization.\textsuperscript{3} Since the morphology of individual TT structures appears typically as blurred fluorescence signal in conventional light microscopy, this may preclude understanding of the local nature of membrane changes in diseased cells, particularly of subtle remodeling processes during HF development.

Two-photon microscopy of healthy rat cardiomyocytes has shown a continuous cell-wide TT network composed mainly of transversal components at striations and longitudinal components.\textsuperscript{12} Magnified optical sections of individual TT components appear blurred in 2-photon images.\textsuperscript{12} Furthermore, confocal microscopy is limited by diffraction, which may hinder analysis of small subresolution TT structures or subtle changes in HF.\textsuperscript{3} Despite nanometric resolution, electron microscopy requires invasive histochemical protocols incompatible with live-cell imaging of intact membrane structures as discussed earlier.\textsuperscript{12} Therefore, live-cell imaging at the nanometer scale represents an important yet unattained goal to improve understanding of individual TT structures in E-C coupling, TT associated nanodomains such as caveolae, and changes in HF.

To overcome resolution limitations in imaging of TT structures in intact cells, we used stimulated emission depletion (STED) microscopy.\textsuperscript{13} A major advantage for live-cell imaging, STED was implemented as laser scanning microscope using a fast beam scanner (Online Figure IA).\textsuperscript{14} STED overcomes the diffraction barrier by switching (turning OFF) fluorescent molecules through a transient dark state.\textsuperscript{13} As in conventional confocal microscopy a focused laser excites all fluorescently labeled structures in the focal spot simultaneously therefore subresolution structures are not optically separated (Figure 1A). The diffusion barrier is overcome by switching peripheral fluorescent markers transiently into a dark state, confining the signal only to molecules at the focal center, by a second red-shifted laser beam with a toroidal (doughnut) profile with a central zero intensity (Figure 1B). Increasing the intensity of the STED beam further confines the central signal spot defined as couplons,\textsuperscript{4,6} protein-membrane complexes that locally control the Ca\textsuperscript{2+} release (spark) function of CRU compartments.\textsuperscript{7} Recent models assume that TTs propagate APs from the surface to an estimated 20,000 CRUs throughout an adult ventricular cardiomyocyte during E-C coupling.\textsuperscript{8}

In contrast, embryonic and early postnatal cardiomyocytes are largely devoid of TTs exhibiting slow intracellular Ca\textsuperscript{2+} transients limited by surface diffusion.\textsuperscript{8} Furthermore, regional loss of TTs was associated with delayed, dyssynchronous Ca\textsuperscript{2+} transients in heart failure (HF) in adult ventricular cardiomyocytes from animal models and patient samples (see Online Table IV).
leading to higher, potentially infinite resolution, even resolving molecular scales. Practical limitations concern properties of the fluorescent STED dye in the sample and diffractive behaviors of large muscle cells.

Using STED microscopy with customized optics and calibration routines, we investigated TT membrane structures in living cardiomyocytes with nanometric resolution, confirmed by nanoparticle measurements (Online Figure IB). We show that STED provides a direct quantitative readout of the local TT membrane morphology and its changes during HF development. Progressive TT changes were analyzed side-by-side in samples from sham versus 4-week (4pMI) and 8-week (8pMI) post-MI hearts and through colocalization and expression analysis of TT associated proteins. We unravel a previously not recognized mechanism of proliferative spatial TT remodeling early during HF development resulting in a highly heterogeneous network phenotype. Furthermore, computational modeling of spatial TT and RyR2 cluster reorganization (orphaning) and Ca2+ release dysynchrony in HF confirmed an aggravating mechanism of delayed subcellular Ca2+ release and AP prolongation, which may contribute to electric and contractile dysfunction.

Methods

For the myocardial infarction model, phenotyping, cardiomyocyte isolation, confocal microscopy protocols, and statistics, an expanded Methods section is available in the Online Data Supplement.

**STED Microscopy Image Acquisition**

A custom STED setup was modified for intracellular TT membrane imaging in isolated cardiomyocytes based on an inverted confocal microscope (Online Figure IA). For live-cell STED imaging of TT membranes we tested several dyes, of which di-8-ANEPPS provided (1) bright membrane signals and (2) quiescent cardiomyocytes (see Methods). The STED microscopy laser configuration is summarized in Online Figure IA. Excitation at 490 nm wavelength by a pulsed diode laser (Pico TA 490, Toptica, Munich, Germany) was focused through a 1.4 NA oil objective (PL APO oil 100x, Leica, Wetzlar, Germany). The collected fluorescence was passed through 2 dichroic mirrors, filtered via a 675/60 band pass filter, and recorded by an avalanche photodiode detector (PerkinElmer, Waltham, MA). STED pulses were delivered by a Ti:Sapphire laser (MaiTai, Spectra-Physics, Darmstadt, Germany) operating at 80 MHz emitting at 750 nm. The STED beam was passed through a Vortex phase plate producing a focal doughnut (RPC Photonics, Rochester, NY). Excitation pulses were temporally overlaid with STED pulses by external triggering. Imaging planes were recorded by resonant mirror scanning along the first lateral axis (15.8 kHz, 

![Figure 2. STED nanoscopy shows transversal TT cross sections with subresolution dimensions in living cells. A, The same TT cross section was imaged by confocal and STED mode. STED shows the morphology of the TT cross section at 3.9 μm imaging z depth. Note: images are rotated according to X (horizontal) and Y (vertical) cell orientations as given by Figure 1B. Scale, 200 nm; colors indicate fluorescence intensity. B, Example of a TT cross section where STED resolved the underlying hollow membrane structure at 5.9 μm z depth. Scale, 200 nm. C, Histograms of diameter dimensions X and Y of the same 205 TT cross sections measured by confocal (upper) versus STED (lower) mode. Diameters represent full-width at half maximum (FWHM) determined by 2D Gauss fitting (see Methods). Vertical red line marks 220 nm, the confocal resolution limit. D, Same TT cross-section pairs as in A and B analyzed by 50% intensity threshold contour algorithm (see Methods). Graphs represent paired contour data each for confocal (black) and STED (red) mode. Note: only STED detected the underlying TT membrane morphology. E, Two-dimensional probability histograms of 205 contours each for confocal and STED mode (gray dashed lines; see legend in figure). STED imaging resulted in a significant leftward shift toward smaller radius sizes confirming faithful detection of subresolution structures (P<0.05). Furthermore, Gaussian fitting confirmed that STED (red line) detected a wider distribution of smaller TT radius sizes as compared with confocal imaging (black line). Mean data are presented in Table 1.](http://circres.ahajournals.org/)

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Quantitative STED Image Analysis of TT Cross-Sections

A 2D Gauss function was fitted to the fluorescence signal distribution of individual transversal TT cross-sections. Full width at half-maximum (FWHM) was determined for longitudinal (X) and transversal (Y) cell directions as indicated in Figure 1B. TT cross-section area was calculated by formula: \( A = \pi \times (0.5X)(0.5Y) \). (2) For the same TT cross sections, contour lines were computed with a resolution of 120 points using a 50% threshold of the maximal pixel intensity. Using the center of mass, contour lines of a given TT population were superimposed in space (X, Y), presented as 2D probability histogram. Furthermore, the mean circumference of individual TT cross-section contours are reported (Tables). (3) The radius size of individual TT cross sections was determined as the median of 120 contour radii and independent of cell orientation. Radius size distributions were plotted. Using a nonparametric test (Mann-Whitney), significant changes in median distribution are reported, and 2-peak Gaussian fitting was used to document deviations (increased heterogeneity) from symmetrical distributions. (4) A separate group of superenlarged highly irregular TT cross sections (Figure 3C) was characterized by manual contour analysis and not included in the automated contour data as detailed in the corresponding Results section. For further details, please refer to the Online Methods.

Skeletal and Spatial Orientation Analysis

Two-dimensional skeletons of intact TT networks were extracted from STED images by ImageJ (image.nih.gov) and Fiji (pacific.mpi-cbg.de plugins). The following analysis steps were applied: local contrast enhancement (CLAHE), median filtering, automated thresholding, and “skeletonizing” of the binary image. TT skeletons were analyzed by Fiji plug-in algorithm (“directionality”) to quantify spatial orientations of network components. Histogram analysis of orientation probability of TT network components relative to principal cell directions is based on local gradient orientations detected by a 5x5 Sobel filter. Network complexity is analyzed through the amount of triple junctions: STED and confocal imaging data are compared in Online Figure VIII.

Computational Modeling of Local Ca\(^{2+}\) Release Function

Local CRU function was investigated through Ca\(^{2+}\) sparks, Ca\(^{2+}\) transients, [Ca\(^{2+}\)]SR, and AP behavior using the fully stochastic mathematical model of Williams et al.\(^8\) which includes spatial determinants of individual CRU nanodomain organization and a realistic number of 20 000 independent release sites. The whole-cell model examines changes in local subspace [Ca\(^{2+}\)] signaling in HF by implementing previously established changes in ion transport proteins (eg, NCX and SERCA; for details see Online Methods). Orphaning of RyR2 clusters during HF due to heterogeneous TT and/or RyR2 cluster remodeling was implemented for only 25% of CRUs through a 30-fold increase in subspace volume, whereas 75% of CRUs remained unchanged.

Results

Nanometric Mapping of TT Membranes in Living Cells

After staining with the membrane probe di-8-ANEPPS (for selection of STED dyes see Online Methods), the central cardiomyocyte region of interest was sequentially imaged by confocal and STED mode and sarcomere orientation (strikations) were documented by transmitted light (Figure 1C). Compared with confocal imaging (Figure 1C), STED immediately revealed sharper TT structures including membrane structures deep inside living cardiomyocytes and individual cross-sections of transversal TTs relevant during E-C coupling (Figure 1C; arrowhead).

Figure 2A shows a representative TT cross-section where STED produced a sharper image of the membrane morphology where the lumen was not resolved due to object limitations, whereas Figure 2B shows a TT cross-section where the interior was resolved confirming the underlying hollow membrane structure. To assess the TT dimensions in situ, we analyzed perpendicular TT diameters corresponding to the longitudinal (X) and transverse (Y) cell orientations as full width at half maximum (FWHM; Figure 1C defines X and Y orientations applied henceforth; see Methods). On average, longitudinal TT diameters (n = 205) from healthy control cells measured 198.7 ± 2.8 nm by STED mode at 3- to 8-μm imaging depth (Table 1). In contrast, the same TTs measured by confocal mode appeared significantly larger (265.9 ± 2.1 nm; P < 0.001). Consistent with sharper STED images, the TT cross-section area appeared 44% smaller when imaged by STED as compared with confocal mode (Table 1).

We wondered if the dimensions of TT cross sections are differently distributed between confocal and STED measurements. Figure 2C shows that the majority of longitudinal (X) and transversal (Y) TT diameters appeared smaller by STED (lower histograms) as compared with confocal imaging (upper histograms), consistent with smaller mean cross-section dimensions (Table 1). Furthermore, similar distributions of TT dimensions were confirmed for STED less than 3 μm near the surface membrane (Online Figure IIA). Notably, for confocal measurements a brisk cutoff in TT diameter sizes occurred at 220 nm (Figure 2C), the confocal resolution limit.

To characterize the local morphology of TT cross sections, we developed an automated 2D contour analysis (see Methods). Figure 2D shows paired contour examples from the same TT cross-sections each for Figure 2A and 2B. Apparently, STED imaging resulted in realistic, detailed contour shapes (red) as compared with confocal mode (black). Figure 2E shows contour overlay plots of the same 205 cross sections (analyzed in Figure 2C) as 2D probability histogram each for confocal and STED imaging. Compared with confocal imaging, STED resulted in a dramatically different probability distribution of TT contours enclosing a smaller area, consistent with a significantly decreased mean circumference (Table 1). Next, we calculated the radius size from individual contours (see Methods) and analyzed the distribution of all median radius sizes for confocal and STED imaging (Figure 2F, gray dashed lines): the radius size distribution shows a significant leftward shift for STED imaging (P < 0.05). In addition, Gaussian fitting confirmed a significantly left-shifted peak distribution (Figure 2F, red line). Furthermore, we found similar contour and radius distributions for subsurface TT cross sections (Online Figure IIB and C, and Online Table I).

In summary, STED captures TT cross-sections with greater accuracy providing sharper images of individual TT cross sections, which can be directly quantified and characterized for representative cell populations.
Figure 3. Transversal TT cross sections in intact cardiomyocytes are progressively enlarged through heterogeneous remodeling during HF development. A, Comparison of striation-aligned STED images showing intracellular TT structures from sham, 4pMI, and 8pMI cells (bottom triangles indicate position of striations). TTs appear enlarged and misaligned in 4pMI and 8pMI cells. Scale, 1 μm. B, STED examples of TT cross sections from sham and 8pMI cells. Scale, 200 nm. C, Confocal versus STED images of a super-enlarged TT cross section complex at 4pMI (triangle; position of striation). Scale, 200 nm. D, Longitudinal (X) and transversal (Y) diameters of TT cross sections were determined (see Methods). Bar graphs summarize mean TT diameters X and Y and cross-section area; right, change in TT cross-section dimensions normalized to sham. *P<0.05 versus sham; †P<0.05 versus 4pMI. E, Two-dimensional probability histograms of contours from TT cross-sections of indicated treatment groups (same cross-sections as in D). TT cross-sections were analyzed by automated contour algorithm (see Methods). Colors represent high (white) versus low (black) contour pixel probabilities as indicated. F, Difference integrals were calculated for radius size distributions between the indicated treatment groups. Radius sizes were determined from individual TT contours (see Methods); for example, at 4pMI-sham, a decrease of small versus an increase of large TT radius sizes occurred during HF development. G, Median radius size distributions were calculated from individual TT contours of sham and 8pMI cells (gray dashed lines). The 8pMI distribution is significantly right-shifted (*P<0.05). Furthermore, 2-peak Gaussian fitting of the 8pMI data (red line) confirmed a rightward shift and an additional second peak documenting heterogeneous changes of TT cross-sections. Mean data are summarized in Table 2.
Table 1. Intracellular TT Dimensions of Untreated Control Cardiomyocytes

<table>
<thead>
<tr>
<th>Imaging Mode</th>
<th>Diameter X, nm</th>
<th>Diameter Y, nm</th>
<th>Area, 10^3 nm^2</th>
<th>Circumference, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confocal</td>
<td>265.9±2.1</td>
<td>261.1±2.0</td>
<td>54.6±0.6</td>
<td>918.9±5.9</td>
</tr>
<tr>
<td>STED</td>
<td>198.7±2.9*</td>
<td>195.2±2.6*</td>
<td>30.8±0.7*</td>
<td>672.9±8.7*</td>
</tr>
</tbody>
</table>

TT cross sections were imaged by confocal and STED modes (at 3 to 8 μm depth). TT diameters represent FWHM measured by automated 2D Gauss fitting (see Methods) for X (longitudinal) and Y (transverse) directions as indicated in Figure 1A. Circumference was determined by automated contour analysis (see Methods). Data represent paired measurements.

*P<0.001 versus confocal imaging; mean±SEM from 205 randomly selected TT cross sections of 43 cells.

Myocardial Infarction Leads to Enlarged TT Cross-Sections

In chronic HF, global changes of the TT network have been documented in living cells by confocal microscopy showing a loss of the transverse component at Z-line striations. So far, neither individual TT structures in living cells, nor quantitative side-by-side comparison of TT changes during different time points of HF development have been quantitatively characterized. Therefore, we investigated transversal TTs each 4 time points of HF development have been quantitatively characterized (see Methods). Figure 1A. Circumference was determined by automated contour analysis (see Methods). Data represent paired measurements.

Table 2. Intracellular TT Cross-Section Dimensions 4 Weeks or 8 Weeks After Myocardial Infarction Compared With Sham Control Treatment

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Diameter X, nm</th>
<th>Diameter Y, nm</th>
<th>Area, 10^3 nm^2</th>
<th>Circumference, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>198.4±1.9</td>
<td>195.5±1.9</td>
<td>30.6±0.4</td>
<td>656.5±5.6</td>
</tr>
<tr>
<td>4pMI</td>
<td>213.0±3.4*</td>
<td>219.9±3.3*†</td>
<td>37.2±0.9†</td>
<td>748.9±11.2††</td>
</tr>
</tbody>
</table>

TT cross sections were imaged by STED deep inside cardiomyocytes each for sham, 4pMI, and 8pMI treatment groups. TT diameters represent FWHM measurements by automated 2D Gauss fitting corresponding to X (longitudinal) and Y (transverse). Circumference was determined by automated contour analysis. Data represent unpaired measurements.

*P<0.05 versus sham; †P<0.05 versus 4pMI; mean±SEM from the following numbers of TT cross sections (cells) for each treatment group: sham, 627 (90); 4pMI, 237 (44); and 8pMI, 290 (46).

We analyzed TT cross-sections by contour method and summarized the data for each treatment group by 2D histogram. TT cross-sections from sham hearts showed a symmetrical, concentric probability distribution characterized by a circular, uniform crest (Figure 3E, sham). However, at 4pMI and 8pMI, both the high probability crest distribution and the periphery of the 2D probability histograms were increasingly altered as reflected by a less uniform distribution and increasing heterogeneity (Figure 3E). We further analyzed contour changes through the difference in radius size distribution for the different treatment groups. Difference integrals revealed a prominent loss of smaller and an increase of larger cross-section radius sizes each at 4pMI and 8pMI versus sham (Figure 3F). Furthermore, a loss of intermediate radius sizes occurred at 8pMI versus 4pMI (Figure 3F). In addition, we determined the median radius size distribution of TT cross-sections for sham and 8pMI. As compared with sham, at 8pMI the median distribution was blunted and significantly right-shifted toward larger radius sizes (Figure 3G, gray dashed lines; P<0.05, Mann-Whitney test). Furthermore, 2-peak Gaussian fitting confirmed heterogeneous changes through a second peak at ~180 nm, indicating a population of TT cross-sections with dramatically increased radius sizes at 8pMI (Figure 3G).

We summarize that the physiological in situ morphology of TT cross-sections is progressively altered during HF development and replaced by an increasingly heterogeneous population of TT components with increased sizes. Furthermore, due to limitations with the automated analysis of complex superenlarged TT morphologies (eg, Figure 3C), we performed a manual contour analysis to estimate cross-section dimensions. At 8pMI, the mean circumference of superenlarged TT complexes was 1458±51 nm (n=40 from 24 cells), a 2-fold increase compared with sham (Table 2; P<10^-10). In addition, we observed superenlarged TT cross sections at subsurface locations at 8pMI, confirming a cell-wide phenotype of heterogeneous proliferative remodeling (Online Figure VA and B). Although many superenlarged TTs approached micrometer dimensions, only STED imaging was able to resolve the underlying membrane morphology of complex luminal configurations.
(Figure 3C) and supported further quantitative analysis of superenlarged TT cross-sections within intact networks.

**STED Reveals Proliferative Network Remodeling After Myocardial Infarction**

Originally, a regional loss of TTs has been described in different HF models (see Online Table IV for overview). Confocal imaging of cardiomyocytes from hypertensive rats with congestive HF showed a decrease in transversal TT network components at \( \approx 19 \) months of age.\(^3\) However, TT network properties were evaluated through the striation-associated signal periodicity,\(^3\) an indirect strategy not addressing individual or irregular structures. Using STED imaging, we directly characterized intact TT networks comprehensively through all local component orientations and segment lengths (see Methods). Cardiomyocytes from sham control hearts showed rectilinear network architectures composed of transversal and longitudinal components (Figure 4A, sham). Accordingly, all-component orientation analysis from sham cells resulted in a probability distribution with 2 peaks corresponding to longitudinal (0°) and transversal (90°) network orientations (Figure 4B, left). In contrast, at 4pMI the TT network showed differential changes of the major network components (Figure 4B), a relative increase in longitudinal (0°) versus a decrease in transversal (90°) components (Figure 4C, 4pMI-sham). In addition, at 8pMI a relative increase in oblique and a reduction of longitudinal components occurred (Figure 4C, 8pMI-4pMI). Gaussian fitting of the longitudinal (0°) and transversal (90°) histogram peaks (Figure 4B, dashed line) confirmed replacement of physiological rectilinear by oblique components at 8pMI, documented by wider 0° and 90° peaks (Figure 4B). Thus, early remodeling of the TT network during HF development at 4pMI is characterized by relative changes of longitudinal versus transversal components, whereas late HF changes at 8pMI are characterized by relative changes in longitudinal versus oblique components. Overall, the TT network appears increasingly fractured and misaligned at striations.

To further explore the prevailing hypothesis of a regional loss of network components (Online Table IV), we analyzed the total length and complexity of intact network skeletons. Surprisingly, we found a significantly increased total network length both at 4pMI and 8pMI (Figure 4D, left), seemingly contrasting with earlier reports and with our own STED observation of sarcomere regions devoid of TT components (Figure 4A, 8pMI). Notably, the total amount of longitudinal and oblique components if directly measured through individual segment lengths was significantly increased, but the total amount of transverse components was unchanged (Figure 4D, center). Furthermore, we found a nearly 2-fold increase in triple junctions consistent with increased post-MI network branching and complexity (Figure 4D, right). Manual contour analysis confirmed an increase in superenlarged TT cross sections at 8pMI (Figure 4A, 8pMI; asterisks). Consistent with Figure 3C, the underlying membrane morphology of superenlarged TT structures was only resolved by STED (Figure 4A and 8pMI), including a significant increase of triple junctions only detected by STED (Online Figure VIII). Therefore, our data suggest increases in TT network length, complexity, and TT dimensions through additive, proliferative membrane remodeling processes early during HF development.

Furthermore, network fracturing and sarcomere misalignment at 8pMI indicate severe disorganization and loss of local TT associations with CRUs at striations, consistent with earlier studies showing a loss of striation associated TTs at relatively late stages of HF (Online Table IV).

**Remodeling of TT-Associated Nanodomains**

During muscle development, TTs and caveolae exhibit membrane interactions, depending on the muscle-specific caveolin-3 (Cav3) isoform.\(^17\) Both Cav3 and junctophilin-2 (Jph2) proteins showed punctate, yet different colocalization behaviors with RyR2 clusters at striations (Figure 5A, sham), consistent with reported nanodomain structures.\(^18\) To directly confirm caveolae like TT nanodomains in living adult cardiomyocytes, we used 3D STED imaging (z-stack) of transversal TTs and identified local membrane evaginations in cross-sections and 3D reconstructions reminiscent of caveolae (Figure 5B and Online Figure VIA through D). Furthermore, Cav3-positive longitudinal structures between striations were increased after MI (Figure 5A, 4pMI; and Online Figure VII), consistent with the documented increase in longitudinal TT components (Figure 4D). The protein expression of Cav3 was significantly increased in ventricular cardiomyocytes at 4pMI (Figure 5D). Although the lumen of TT evaginations was not resolved at the given resolution of \( \approx 60 \) nm (Online Figure IB), the membrane structures are consistent with the dimensions of caveolae. Whereas 3D reconstructions from sham cardiomyocytes showed spatially separated transversal TT structures at neighboring striations (Online Figure VII), 8pMI cells often showed membranous connections between transversal TTs through longitudinal components and abnormally branched, superenlarged cross-section structures (Online Figure VIF). The complex TT network morphology at 8pMI together with the observed increase of Cav3-positive structures suggests a mechanism of proliferative remodeling through longitudinal and oblique TT growth as documented by component analysis during HF development (Figure 4D).

Notably, the localization of RyR2 clusters was significantly changed at 4pMI (Figure 5A, lower left), as documented by a significant decrease of the striation associated power function of the first periodic peak (Online Figure VII; sham \( n = 6, 4pMI \ n = 5 \) cells), indicating a loss of RyR2 clusters at striations. Furthermore, higher power values between periodic peaks indicated an increase of RyR2 clusters at atypical locations (Online Figure VII), consistent with post-MI spatial reorganization and orphaning. Because post-MI RyR2 protein expression was not changed (Figure 5D and data not shown), this suggests spatial reorganization of RyR2 clusters as possible orphaning mechanism. Furthermore, Jph2 signals appeared altered compared to RyR2 at 8pMI (Figure 5, lower right), and the striation associated peak power was significantly decreased (Online Figure VII). In agreement, heart-restricted inducible shRNA knockdown of Jph2 resulted in significantly increased variability of CRU spacing at TT junctions,\(^19\) and Jph2 knock-out showed that the SR transmembrane protein stabilizes CRU nanodomains through a cytosolic phospholipid binding domain and colocalization with LCC and RyR2 membrane complexes.\(^20\)

We confirmed colocalization of Jph2 and RyR2 clusters (Figure 5A, top right), which was altered at 8pMI (Figure 5A, lower right).
Post-MI expression of Jph2 was significantly decreased (Figure 5D), to a similar degree as in conditional Jph2 shRNA knockdown hearts with increased RyR2 cluster/TT spacing and abnormal Ca\textsuperscript{2+}/H\textsubscript{11001} release. Therefore, we investigated changes in Ca\textsuperscript{2+}/H\textsubscript{11001} release both by combinatory (TT and Ca\textsuperscript{2+}) and orientation differentiating strategies (transversal versus longitudinal confocal line scanning). As expected, sham cells showed widely spaced longitudinal TT signals between striations in transverse line scans with synchronous onset of Ca\textsuperscript{2+} release, analyzed by time to F50 threshold, resulting in a low dyssynchrony index (Figure 5C, sham). In contrast, transverse line scans of 4pMI cells showed an increase of longitudinal/oblique TT signals between striations and heterogeneously delayed Ca\textsuperscript{2+} release, resulting in a high dyssynchrony index (Figure 5C, 4pMI). On average, sham cells showed the same low dyssynchrony index of 2.2±0.1 ms for transversal and longitudinal line scans, whereas 4pMI cells showed delayed Ca\textsuperscript{2+} release and in-
creased dyssynchrony indexes both in transverse (6.7 ± 1.2; n = 11) and longitudinal (5.0 ± 1.5; n = 9) directions. As the most profound change in dyssynchronous Ca\(^{2+}\) release delay occurred between transversal striations with increased longitudinal/oblique TT components (Figure 4) and we found reorganization of RyR2 clusters at striations, we next investigated RyR2 orphaning as a spatial mechanism of Ca\(^{2+}\) release dysfunction using a recent CRU nanodomain model.\(^8\)

**Computational Modeling of Spatial CRU Changes**

Our data suggest heterogeneous changes of individual TT components as well as RyR2 clusters and CRUs. The findings are consistent with and extend the previously described RyR2 “orphaning”\(^3\) and further show decreased Jph2 levels previously established as mechanism of increased CRU spacing at TT junctions.\(^19\) To assess the impact of increased, yet heterogeneous spatial CRU changes in HF, we used an advanced computational model based on 20,000 individual Ca\(^{2+}\) release sites, a realistic estimate for ventricular cardiomyocytes.\(^8\) Heterogeneous TT changes were investigated through increased spacing between TTs and RyR2 clusters at CRUs of only 25% of TT junctions (whereas 75% of CRU nanodomains remained unchanged), consistent with our data and earlier studies.\(^3\) HF was investigated by implementing previously established changes in ion transport (NCX, SERCA; see Methods). Under steady-state pacing conditions (1 Hz), the CRU model readily reproduced AP prolongation in HF, which was further worsened by additional RyR2 orphaning, whereas isolated RyR2 orphaning (without any HF changes) at 25% of CRUs caused only minimal AP prolongation (Figure 6A). In HF, AP prolongation was paralleled by a delayed cytosolic [Ca\(^{2+}\)] load in diastole (Figure 6B). Importantly, the CRU model reproduced decreased SR Ca\(^{2+}\) load, a hallmark of E-C coupling pathophysiology in HF, which was blunted by RyR2 orphaning (Figure 6C). Unexpectedly, combined RyR2 orphaning with HF normalized SR [Ca\(^{2+}\)] load in diastole (Figure 6C). Furthermore, in HF depletion of diastolic SR [Ca\(^{2+}\)] load resulted in an increased late L-type Ca\(^{2+}\) current (LCC) due to decreased Ca\(^{2+}\) dependent inactivation, further increased by RyR2 orphaning in HF (Figure 6D).

We investigated the local CRU function through total peak RyR2 Ca\(^{2+}\) release flux, which was strongly decreased in HF, contributing to a delayed [Ca\(^{2+}\)] transient and an abnormal second peak in diastole (Figure 6E). Both decreased peak RyR2

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**Figure 5. Remodeling of TT-associated nanodomains, Ca\(^{2+}\) release dyssynchrony, and protein expression in early HF (4pMI).**

**A.** top. Sham cardiomyocytes show differences in Cav3 versus Jph2 colocalization with RyR2 clusters as evidenced by color separation of punctate Cav3 and RyR2 signals at striations (para-localization), whereas Jph2 and RyR2 are fully colocalized (yellow puncta). Note Cav3 signal (red) rarely occurs between striations. Dotted rectangles correspond to magnification.

**B.** left, STED image of neighboring TT cross sections, only the right TT shows a membrane evagination; right, rotated 3D reconstruction of the same TT reveals abrupt change in morphology of the right TT. Triangle indicates position of leftward imaging plane; scale, 200 nm; see Online Figure VI for extended data.

**C.** Simultaneous imaging of Ca\(^{2+}\) and TT signals by transversal line scans of sham and 4pMI cardiomyocytes. Ca\(^{2+}\) release synchrony was quantified by half-maximal thresholding (F50) and temporal variability analysis (leading edge behavior) similar to Louch et al.\(^10\) The dyssynchrony index is 2.2 ms for sham and 15.6 ms for 4pMI. For average dyssynchrony indexes of transversal and longitudinal scans, see Results section. **D.** RyR2, Jph2, and Cav3 immunoblots from cardiomyocytes isolated from sham, 4pMI, and 8pMI hearts as indicated. Bar graphs summarize change in protein expression normalized to sham from at least 3 independent measurements; *P < 0.05 versus sham; n.s. indicates not significant.
Ca\(^{2+}\) release flux and delayed \([Ca^{2+}]_i\) release in diastole corresponded with prolonged AP repolarization, which was further delayed by RyR2 orphaning (Figure 6A and 6E). Moreover, the early systolic integrated RyR2 Ca\(^{2+}\) flux was blunted in HF, which was further decreased by RyR2 orphaning (Figure 6F). In diastole, a dramatic increase in late RyR2 Ca\(^{2+}\) release flux occurred during AP repolarization and was further increased by RyR2 orphaning (Figure 6F). Furthermore, after completion of AP repolarization, increased diastolic RyR2 Ca\(^{2+}\) leak persisted as evidenced by an increased spark rate and spark duration in HF (Figure 6E; inset). Whereas RyR2 orphaning by itself did not increase spark dependent Ca\(^{2+}\) leak, in HF additional orphaning of RyR2 clusters further increased diastolic Ca\(^{2+}\) sparks (Figure 6E; inset) and Ca\(^{2+}\) leak (Figure 6F). Thus, RyR2 orphaning as occurs during heterogeneous spatial TT and CRU reorganization in HF resulted in macroscopic AP prolongation explained by a pronounced delay of SR Ca\(^{2+}\) release late in diastole and in a blunted systolic Ca\(^{2+}\) transient. Therefore, in addition to E-C uncoupling in HF, heterogeneous spatial reorganization of TTs and RyR2 clusters may aggravate the experimentally observed delayed and dysynchronous SR Ca\(^{2+}\) release, promote severe diastolic SR Ca\(^{2+}\) leak, and potentially contribute to afterdepolarizations and Ca\(^{2+}\)-triggered arrhythmias.

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Earlier live-cell studies in models of late stage HF showed either a regional loss of TT density or spatial network reorganization.\(^3\) Because the dimensions of individual TTs were below the resolution limit of conventional light microscopy,\(^4\) we have established noninvasive super-resolution imaging of intact TT membrane structures deep inside living cardiomyocytes, based on the STED concept.\(^5\) In addition, we developed quantitative image analysis strategies which showed circular morphologies of intact TT cross-sections in situ with diameters of \(\approx 200\) nm. Based on these strategies, we investigated the hypothesis that TT structures undergo early, progressive remodeling during HF development. Using STED, we provide direct in situ evidence of TT remodeling through heterogeneous enlargement of cross-sections and an increase of TT network length, establishing (1) an early time point (4pMI) of TT remodeling during HF development and (2) a proliferative mechanism of membrane reorganization. In particular, we show by quantitative analysis that the rectilinear TT network was differentially remodeled during HF development at 4pMI through significantly increased longitudinal and oblique components. The TT changes deteriorated rapidly during HF development resulting in a heterogeneous, fractured and misaligned TT network at 8pMI. In addition, Jph2 and Cav3 protein levels were significantly changed during HF development, each indicating altered TT associated CRU and caveolae nanodomain functions, respectively. Finally, Ca\(^{2+}\) imaging, analysis of RyR2 clusters, and computational modeling of heterogeneous TT and RyR2 orphaning changes uncovered spatial mechanisms of Ca\(^{2+}\) release delay and AP prolongation during diastole and in a blunted systolic Ca\(^{2+}\) transient. Therefore, in addition to E-C uncoupling in HF, heterogeneous spatial reorganization of TTs and RyR2 clusters may aggravate the experimentally observed delayed and dysynchronous SR Ca\(^{2+}\) release, promote severe diastolic SR Ca\(^{2+}\) leak, and potentially contribute to afterdepolarizations and Ca\(^{2+}\)-triggered arrhythmias.

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in HF. To our best knowledge, this is the first super-resolution study of membrane structures in cardiomyocytes establishing live-cell STED imaging as direct quantitative strategy to investigate TT membranes in healthy and diseased cells.

This study overcomes the diffraction barrier deep inside large excitable cells, using STED nanoscopy and the membrane probe di-8-ANEPPS. We have carefully evaluated different STED-compatible membrane dyes to ensure effective staining and cardiomyocyte integrity. This information is essential to achieve sufficient fluorescence membrane signals from the typical attoliter-sized spot volumes used by STED nanoscopy (Figure 1B). STED has previously shown intracellular antibody tagged vesicles in living neurons\(^{14}\) and has resolved YFP-tagged ER structures in PtK2 cells.\(^{21}\) However, YFP expression strategies require prolonged cell culture, which promotes TT loss in cardiomyocytes, and neither are antibody-based strategies advantageous to delineate TT membranes. In addition, we have shown that STED is not limited by intracellular imaging depth by comparing subsurface and deep intracellular imaging data and through 3D reconstruction of TT membrane invaginations. Nanoscale imaging of even smaller TT substructures such as caveolae is limited by the given lateral and axial resolutions of \(\approx 60\) nm and \(\approx 500\) nm, respectively, which could potentially be improved by additional instrumentation such as combining STED with 4Pi microscopy\(^{22}\) or by additional STED beams for further resolution enhancement. Other super-resolution methods such as STORM and (f)PALM depend on time-multiplexed localization of random signals from single molecules and image reconstruction.\(^{23}\) In comparison, for freely diffusing membrane dyes, STED nanoscopy might be advantageous as local membrane signals are directly characterized. Compared with STORM or STED, structured illumination is limited by a maximal 2-fold resolution increase.\(^{23}\) We conclude that STED nanoscopy has clear advantages for studies of subresolution membrane structure and disease changes (Online Figure VIII), even for deep intracellular imaging of individual TT structures in living cells and under challenging imaging conditions in large muscle cells with densely packed disfactive myofilaments and mitochondria. STED nanoscopy together with the presented analytic framework of quantitative TT nanopathology may therefore open avenues for future translational studies of human HF samples and pathophysiological interpretation through temporally controlled studies of progressive heart disease development.

Considering existing concepts of TT changes (for overview see Online Table IV), regional loss of TTs remains an important model. Furthermore, spatial network reorganization has been proposed as E-C uncoupling mechanism.\(^{3}\) Electron microscopy studies of cat hearts have estimated TT cross-section dimensions at 150 to 200 nm, depending on fixation methods,\(^{24}\) which is clearly below the resolution limit of conventional light microscopy as confirmed by our confocal imaging studies of TT cross-sections (Figure 2). In contrast, using noninvasive super-resolution techniques, we have directly determined the in situ dimensions of hundreds of TT cross-sections from healthy cells with mean dimensions approximating 200 nm (Table 1 and Online Table I). Furthermore, contour analysis and optical sectioning established cylindrical TT membrane structures in healthy cells (Figures 2 and 5 and Online Figure VI). Based on typical in situ TT dimensions and circular cross-section morphologies, we propose that TTs function as “nanowires” during intracellular AP propagation in muscle cells. If intracellular AP propagation depends on local TT properties, then maintenance of membrane integrity and physiological network structures is essential to ensure synchrony of E-C coupling at an estimated 20,000 CRUs. Indeed, combined Ca\(^2+\) and TT imaging of 4pM I myocytes showed increased spatial heterogeneity of intracellular Ca\(^2+\) release between striations with increased longitudinal/oblique TT components. This suggests that dysynchronous Ca\(^2+\) release, reported earlier in HF,\(^{3,10,11,25}\) might be caused by TT proliferation and membrane reorganization of CRU nanodomains. Furthermore, we find significant reorganization of RyR2, Jph2, and Cav3 clusters after MI, representing distinct TT associated nanodomains and potential RyR2 orphaning mechanisms.

Consistent with earlier observations in late-stage HF,\(^{3}\) we found a relative decrease of transversal network components at 8pMI (Figure 4). In addition, STED imaging established an early network phenotype at 4pMI, characterized by significantly increased longitudinal components reminiscent of immature cells.\(^{9}\) Although an increase of longitudinal network components has been documented during intermediate\(^{26}\) and late\(^{1}\) HF stages by confocal imaging, STED identified a previously unknown significant increase in longitudinal TT components already 4 weeks after infarction, based on sharper images and direct component analysis (Online Figure VIII). To characterize local TT signals from STED images, we used a novel strategy of quantitative analysis and found significant increases of the total amount of longitudinal and oblique TT components at 4pMI (Figure 4D). This raises the possibility that additive membrane processes and/or reactivation of fetal genes contribute to early TT remodeling during HF development. Previous work established an immature embryonic TT system not connected to the surface membrane and SR terminals until completion of postnatal maturation through transverse TT invaginations.\(^{9}\) Our data showing a total increase in TT network length through longitudinal and oblique components during HF development (Figure 4D) are consistent with a recent confocal study showing less uniform and often oblique TTs with increased dimensions in human HF\(^{27}\) and with earlier electron microscopy examples of failing and hypertrophied human hearts describing TT “proliferation” and increased dimensions.\(^{28}\) Electron microscopy studies showed that the majority of longitudinal tubules in healthy rat myocytes form SR junctions with ultrastructural features similar to couplons, suggesting the potential for E-C coupling and Ca\(^2+\) release.\(^{29}\)

Junctional complexes stabilize CRUs between terminal SR release sites and TTs through lipid-protein interactions. Jph2 bridges the junctional cleft through interactions with phospholipids at TT membranes.\(^{20}\) We found significant Jph2 downregulation early in HF comparable in extent with heart-specific shRNA targeted Jph2 knockdown, which resulted in TT remodeling with increased CRU spacing and Ca\(^2+\) leak.\(^{19}\) After MI, we showed significant reorganization of Jph2 and RyR2 clusters. We note that Ca\(^2+\) release dysfunction can by itself accelerate cardiac remodeling as evidenced earlier under comparable HF conditions.\(^{16}\) Furthermore, we found a significant early increase in Cav3 expression together with a significant increase of longitudinal TT components early during HF development. Cav3 overexpression has been
shown to induce cardiomyopathy through a dystrophin-dependent mechanism. We note that cardiomyocytes are exposed to abnormal stretch conditions during dilative HF (Online Figure III) and have previously shown that abnormal load conditions lead to differential activation of embryonic gene programs. Since Cav3 is trafficked through vesicle carriers to TT membranes via the Golgi complex, it is strategically positioned to directly participate in TT membrane remodeling. We documented para-localization of Cav3 with RyR2 clusters at transversal striations, a pattern recently shown at the cardiomyocyte surface during revision of this manuscript. Therefore, post-MI changes in Cav3 expression may influence para-localized RyR2 clusters, for example, through recruitment and stabilization of cholesterol-enriched glycolipid rafts, repeated caveolae formation, proliferation of TTs, and possibly altered aggregate behaviors of RyR2 cluster proteins. We speculate that the observed increase in Cav3 expression during TT network remodeling at 4pMI represents an adaptive response during hypertrophic remodeling (Online Figure IV). Therefore, it will be particularly important to extend our observations to molecular Cav3 mechanisms of TT remodeling in future studies.

We used computational modeling to investigate a realistic, massive number of functionally independent CRUs, a prerequisite to characterize CRU changes, which we found during heterogeneous TT remodeling, and earlier studies during orphaning of RyR2 clusters in HF or during HF induced by Jph2 knockdown. Our data show that heterogeneous CRU spacing and RyR2 orphaning on top of global HF changes aggravated the phenotype through delayed SR Ca\(^{2+}\) release and AP prolongation. Unexpectedly, HF with RyR2 orphaning resulted in normalization of diastolic SR [Ca\(^{2+}\)] load explained by reduced LCC current inactivation due to increased spacing of RyR2 release sites (Figure 6D and Online Figure VII). Normalization of SR load is in agreement with a previous study in a pig model of ischemic heart disease that found even increased SR Ca\(^{2+}\) load based on NCX current measurements. Normalized SR load in HF with RyR2 orphaning contributed to a more pronounced delayed Ca\(^{2+}\) flux delay and to increased diastolic SR Ca\(^{2+}\) leak through abnormally increased spark activity. Different from Williams et al., an earlier modeling study of LCC gating changes showed delayed Ca\(^{2+}\) signaling. However, we found reduced LCC current inactivation in HF based on individual CRU nanodomains due to increased local heterogeneity of release sites, supporting complex subcellular changes in Ca\(^{2+}\) signaling. Therefore, future super-resolution studies will need to assess local mechanisms of SR Ca\(^{2+}\) leak directly at CRUs, potentially through novel STED dyes or combined imaging techniques.

In summary, we demonstrate that STED imaging provides fundamentally new insight through local information of the TT morphology in living cardiomyocytes that was previously unattainable and show how critical super-resolution studies are to understand the integrity of complex intracellular membrane systems. In addition, STED nanoscopy provided reproducible membrane localization information, which is not subject to potential artifacts from postprocessing or indirect strategies. We have developed novel quantitative analysis strategies which allow unbiased, automated data processing and characterization of individual TTs and network components in living cells. Through STED imaging and molecular analysis, we have uncovered a mechanism of additive TT remodeling consistent with an early HF phenotype of TT proliferation previously not recognized. Our studies further implicate caveolae as TT substructures that may contribute to TT remodeling through membrane trafficking. In addition, significantly decreased Jph2 expression indicates increased CRU spacing in the context of heterogeneous TT membrane changes, a phenotype investigated through computational modeling of heterogeneous CRU changes aggravating delayed Ca\(^{2+}\) release and AP prolongation. STED nanoscopy of living cardiomyocytes will be particularly useful to gain mechanistic insight about the function of TT changes in different heart disease models, to explore early and potentially reversible disease mechanisms with high accuracy, to test interventions which may prevent TT remodeling, and to translate basic mechanisms and concepts through nanopathology studies of patient samples.

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Disclosures

None.

References

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Novelty and Significance

What Is Known?

- Post-myocardial infarction heart failure (HF) is a leading cause of morbidity and mortality that is increasing worldwide.
- Remodeling of the T-tubule membrane system, which has been shown in patients and animals with HF, may represent an important mechanism of pathological remodeling.
- Due to small subresolution dimensions, the imaging of subcellular T-tubule membrane structures may require super-resolution imaging.

What New Information Does This Article Contribute?

- A super-resolution technique called stimulated emission depletion (STED) microscopy shows T-tubule membrane structures with nanometric resolution deep inside living cardiac myocytes for the first time.
- In cardiac myocytes, T-tubule remodeling occurs through an additive, proliferative remodeling process that results in profound membrane network reorganization and Ca$^{2+}$ signaling dysfunction as early as 4 weeks after myocardial infarction in mice.
- Previous studies suggest that the T-tubule system contributes to abnormal Ca$^{2+}$ signaling in animals and patients with HF. We developed novel strategies for super-resolution imaging and quantitative analysis of T-tubules in living cardiac myocytes using STED microscopy. We investigated the nature of T-tubule remodeling based on significantly improved nanometric resolution and show that membrane remodeling underlies subcellular Ca$^{2+}$ cycling defects in HF. We found that individual T-tubule cross sections increase progressively in dimensions over several weeks after myocardial infarction, suggesting additive membrane remodeling processes. The physiological rectilinear T-tubule network organization in mice was found to undergo dramatic reorganization with an increase in length and the addition of new components. The time course and increase of T-tubule membrane components correlated well with an increase in the expression of muscle-specific caveolin-3 proteins that are known to contribute to T-tubule organization in the developing heart. The spatial reorganization of the T-tubule network correlated with increased dyssynchronous intracellular Ca$^{2+}$ release in mice after myocardial infarction. The Ca$^{2+}$ release dyssynchrony is explained by orphaning of ryanodine receptor (RyR2) Ca$^{2+}$ release channels due to disruption of junctional signaling microdomains during T-tubule remodeling. Thus, our data suggest that T-tubule remodeling and orphaning of RyR2 clusters might play important roles in the development of HF by contributing directly to electric and contractile dysfunction.
Stimulated Emission Depletion Live-Cell Super-Resolution Imaging Shows Proliferative Remodeling of T-Tubule Membrane Structures After Myocardial Infarction
Eva Wagner, Marcel A. Lauterbach, Tobias Kohl, Volker Westphal, George S.B. Williams, Julia H. Steinbrecher, Jan-Hendrik Streich, Brigitte Korff, Hoang-Trong M. Tuan, Brian Hagen, Stefan Luther, Gerd Hasenfuss, Ulrich Parlitz, M. Saleet Jafri, Stefan W. Hell, W. Jonathan Lederer and Stephan E. Lehnart

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Supplemental Materials and Methods

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D. Supplemental References
A. Detailed Methods

**Myocardial infarction model and in vivo phenotyping.**

Adult female C57BL/6N mice were randomized to post myocardial infarction (post-MI) remodeling for either 4 weeks or 8 weeks (4pMI or 8pMI, respectively) or sham procedure. The left anterior descending coronary artery (LAD) was proximally ligated. Cardiac remodeling was characterized in vivo by rodent echocardiography (Vevo2100 VisualSonics, Toronto) and by normalized heart weight. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at University Medicine Goettingen and by veterinarian state authority LAVES (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit) in compliance with the humane care and use of laboratory animals. Mice were treated 3 days before, during and 3 days after surgery by buprenorphin (0.06 μg/g body weight s.c.) and metamizol (1.33 mg/ml by drinking water), and during surgery with 2% isoflurane in O₂ ventilated with a tidal volume of 0.15 ml (150 strokes/min). A left lateral thoracotomy was performed, and the left anterior descending coronary artery (LAD) was proximally ligated with a 9-0 polyamide suture. Myocardial infarction was immediately confirmed as permanent distal tissue blanching through a binocular operating microscope, and infarct size estimated (0-100%). Sham operated animals underwent the same procedure, except occlusion of the LAD. The chest was closed, the lungs were inflated, and the animal monitored until spontaneous, regular breathing occurred and daily following surgery.

Pre- and postoperatively mice were characterized by echocardiography (Vevo2100 VisualSonics, Toronto) using a 30 Hz transducer (MS-400 MicroScan Transducer, Linear Array Technology). Left ventricular dimensions were determined by parasternal long axis and short axis views. Echocardiographic dimensions reported include terminal observation time points (Online Figure III) for ejection fraction (EF) and diastolic cross-section area (Area, d) calculated from consecutive parasternal left ventricular contraction cycles. In addition, post-MI scar
thinning of the anterior free wall was confirmed by measuring anterior wall thickness (AWTh,d) and confirmed as significant decrease versus sham (data not shown). At the time of euthanasia wet total heart weight was determined and normalized to body weight (HW/BW). Based on previous experience with the same background strain, same LAD ligation protocol, and histological analysis of transversal heart sections (data not shown), infarct sizes approximate 35% on average at 8pMI. Therefore, infarct size was again estimated (0-100%) during organ extraction and only hearts with consistent infarct sizes were used for further analysis.

Female as compared to male mice were reported to show better post-MI survival attributed to reduced mortality, less dilative remodeling, a reduced incidence of cardiac rupture, and better LV systolic function. As our study investigated early versus late post-MI remodeling mechanisms during progressive heart failure development, female mice were considered advantageous in order to establish continuous uniform data samples at multiple investigatory levels in a relatively stable post-MI population with predictable HF behavior. As expected, the cardiac and cellular pathology each showed significant post-MI progression (Online Figures III and IV) confirming a relatively uniform disease substrate.

**Myocyte preparation for live cell imaging**

Isolated mouse hearts were Langendorff perfused with a Ca$^{2+}$ free oxygenated Krebs buffer followed by collagenase type II containing solution (2 mg/ml, Worthington Biochemical Corporation). Ventricular cardiomyocytes were plated on laminin coated glass cover slips for subsequent imaging studies. For cardiac myocyte isolation mice were euthanized, hearts were quickly extracted and the aorta connected to a 21G cannula. The heart was Langendorff perfused with Ca$^{2+}$ free oxygenated Krebs solution (in mmol/L: NaCl 120.4, KCl 14.7, KH$_2$PO$_4$ 0.6, Na$_2$HPO$_4$ 0.6, MgSO$_4$ 1.2, HEPES 10, NaHCO$_3$ 4.6, taurin 30, 2,3-butanedione-monoxime 10, glucose 5.5, pH 7.4) at 37°C for 4 mins. Perfusion was switched to collagenase type II
containing solution for 9 mins. Following digestion, left and right ventricle tissue was dissected into 1 mm³ pieces in collagenase containing buffer, whereas all post-MI scar tissue was carefully excised and discarded. Digestion was stopped by the Krebs buffer containing 10% bovine calf serum (HyClone Laboratories) and 12.5 µM CaCl₂. Isolated myocytes were washed at least two times before plating on laminin (mouse laminin, BD Biosciences) coated glass cover slips (Ø 42 mm, Thermo Scientific) at a nominal density of 1500 cells/cm².

Selection of membrane probe for live cell STED imaging

We used STED nanoscopy to image the continuous TT membrane network in living cardiac myocytes stained with fluorescent membrane dyes. In pilot studies, we tested several dyes of which di-8-ANEPPS resulted in bright signals of TT membranes in stably quiescent myocytes during STED imaging (Figure 1), an important prerequisite for super-resolution live cell imaging. However, myocytes stained with previously described derivatives of fluorescently labeled phosphoethanolamines (Atto647N-PE and Atto647N-PE1) or sphingolipids (Atto647N-SM)⁷, or di-4-ANEPPS showed different degrees of dye internalization and/or contracted irregularly. Therefore, we performed all imaging experiments using a myocyte staining protocol based on the lipophilic fluorescent probe di-8-ANEPPS (50 µM, Molecular Probes) for 10 mins at room temperature followed by two washing steps with Krebs buffer. Myocytes were randomly selected and imaged in fresh physiological Krebs buffer on laminin coated coverslips.

STED microscopy, image acquisition, and image analysis

Please also refer to the Methods section of the main manuscript.

Image processing for analysis of TT dimensions by FWHM

Prior to quantitative analysis, all images where deconvolved using a Wiener filter and the respective point spread function as kernel (Matlab R2009b; The MathWorks). Next, a 2D Gauss
function was fitted to the fluorescence signal distribution of unconnected ("free") transversal TT cross-sections (Figure 1B, rightward images, marked by arrowhead). The function used different variances along both orthogonal axes. The orientation of the two perpendicular axes was fixed and aligned with the two principal cell axes during fitting (longitudinal or transversal). Cell orientation was determined from transmitted light images as shown in Figure 1B. Accordingly, full width at half maximum (FWHM) was determined along the longitudinal (X) and the transversal (Y) axis from the fitted 2D Gauss data. The corresponding ellipse area was calculated from the measured X and Y diameters for each TT cross-section by formula:
\[
A = \pi \left( 0.5 \cdot X \right) \cdot \left( 0.5 \cdot Y \right).
\]
While FWHM depends on the microscope point-spread-function, we used regular nanobead calibrations (20 nm) to ensure stability between measurements.

**Automated TT cross-section analysis by contours and data processing**

Contour lines were computed with a resolution of 120 points per curve using the Matlab command `contourc` for individual TT cross-sections using a 50% threshold of the maximal pixel fluorescence intensity in deconvolved images. Using the center of mass, all contour lines of a given TT population were superimposed in space (X, Y) and presented as 2D probability histogram (color coded, contour procedures described with Figure 2). In addition, the circumferences of TT contours are reported as mean data by Tables 1 and 2 and Online Tables I and II.

**Radius analysis from individual TT contours**

In addition, we determined the radius size of individual TT cross-sections as the median radius of the corresponding contour. Median distributions are presented for different imaging modes or treatment groups. Using a non-parametric test (Mann-Whitney) changes in median distribution of the radius sizes were accepted for p<0.05. Furthermore, two-peak Gaussian fitting was used to document heterogeneous changes in TT radius sizes 8 weeks post-MI (8pMI).

**Skeleton detection and spatial orientation analysis**
2D skeletons of continuous TT networks were extracted from STED images by ImageJ (imagej.nih.gov) and Fiji (pacific.mpi-cbg.de). A statistical region-merging algorithm was applied, the data were binarised by thresholding of identical intensity percentiles, and continuous ("intact") skeleton data were extracted from the binarised images.\textsuperscript{8} TT skeletons were next analyzed with a Fiji plug-in algorithm ("directionality") to quantify the spatial orientations of individual network components from continuous TT networks. We computed histograms to assess the orientation probability of TT network components in 2D space and corresponding to the two principal cell directions (longitudinal versus transversal) based on local gradient orientations detected by a 5x5 Sobel filter.\textsuperscript{9}

**Cell size and co-immunofluorescence measurements**

Cell area, length and width were documented (Online Figure IV). To determine myocyte dimensions, a fraction of the cells were fixed immediately after isolation with 4% PFA (5 mins at room temperature) following adherence on laminin coated coverslips (mouse laminin, BD Biosciences). Following additional PBS washing steps (pH 7.4, CaCl\textsubscript{2} and MgCl\textsubscript{2} free, GIBCO) cells were embedded in mounting medium (ProLong Gold Antifade Reagent, Invitrogen). Fixed samples were documented by transmitted light mode (Zeiss LSM 710, Jena, Germany). Using ImageJ (imagej.nih.gov) the myocyte surface border/contour, area, length and width were determined as presented in Online Figure IV.

For immunofluorescence studies cells were permeabilized (0.2 % Triton X-100, 10 % bovine calf serum in PBS) for 1 h after the PBS washing steps. Cells were incubated over night with the following primary antibodies and dilutions: rabbit anti-junctophilin-2 1:500 (Invitrogen 40-5300), rabbit anti-caveolin-3 1:500 (Abcam ab2912), and mouse anti-RyR2 1:500 (Thermo Fisher Scientific MA3-916). After three washing steps, cells were incubated for 1.5 hrs with the following secondary antibodies and dilutions: anti-mouse Alexa Fluor 514 1:1000 (Invitrogen A31555) and anti-rabbit Alexa Fluor 633 1:1000 (Invitrogen A21071). Samples were imaged.
using a Zeiss LSM 710 using a 63x 1.4 NA oil objective and a pixelsize of 80 nm. Excitation (ex.) and detection (det.) settings were as follows: Alexa Fluor 514 ex. 514 nm, det. 520 – 620 nm; Alexa Fluor 633 ex. 633 nm, det. 640 – 740 nm.

**Analysis of T-tubule associated protein localizations**

The signal distribution of Cav3, RyR2, or Jph2 immunostained cardiomyocytes was analyzed by Fast Fourier transformation (FFT) using ImageJ (imagej.nih.gov). The power spectrum of each image was computed individually and the average power spectra for the indicated treatment groups (sham, 4pMI, 8pMI) are shown as a function of spatial frequency.\(^{10}\) Data are presented as Online Figure VII.

**Combined confocal Ca\(^{2+}\) and T-tubule imaging in transverse and longitudinal directions.**

Using a LSM 710 confocal microscopy system (Carl Zeiss; Jena, Germany) and a 63x 1.4 NA oil objective, line scan imaging of cardiomyocytes was performed each in the transversal and longitudinal cell direction. Di-8-ANEPPS (10 µM, 10 min) and fluo-4 AM (10 µM, 30 min) signals were recorded by longitudinal scanning at room temperature according to the protocol described by Louch et al.\(^{11}\) Since the LSM 710 system allows for a high spectral separation, di-8-ANEPPS (excitation: 458 nm, detection: 550 - 740 nm) and fluo-4 AM (excitation: 488 nm, detection: 490 – 540 nm) signals were separately recorded. We used a modified protocol using a 20 µm scan line (100 pixels) each for transversal and longitudinal directions to correlate functional measurements with directional post-MI T-tubule component changes (which have been independently documented by STED imaging). Image analysis was performed with ImageJ. The spatially averaged fluo-4 line scan signal was thresholded at 50% intensity, to analyze temporal deviations from F50 as described by Louch et al.\(^{11}\) For transversal line scan imaging, representative T-tubule and Ca\(^{2+}\) signals are presented in Figure 5C. Temporal deviations (standard deviation) from F50 are documented as the dyssynchrony index.
Protein analysis

Isolated cardiac myocytes were used for biochemical analysis, pelleted by sedimentation for 8 min, immediately snap-frozen and stored at -80°C. Cells were homogenized using a 0.5 - 1.0 ml glass/Teflon Potter-Elvehjem homogenizer (300 rpm; 10-20 strokes) in homogenization buffer consisting of: 50 mmol/L Hepes, KOH titrated pH 7.4, 300 mmol/L sucrose, 150 mmol/L NaCl, 0.5 % (v/v) Triton X-100, protease and phosphatase inhibitor mix (Roche). To remove insoluble debris, lysates were spun at 8,000 g for 15 mins at 4°C. The supernatant was aliquoted and immediately snap-frozen. For Western blots 20 µg of protein were resolved by SDS-PAGE using a 4-12% gradient gels (Novex, Life Technologies) and transferred to Protean nitrocellulose membranes (Whatman), blocked in 5% (w/v) nonfat milk in Tris-buffered saline with 0.05% (v/v) Tween 20, and detected using the following antibodies and dilutions: anti-junctophilin-2 1:1000 (Invitrogen 40-5300), anti-caveolin-3 1:2000 (Abcam ab2912), anti-RyR2 1:2500 (Prestige, Sigma), anti-GAPDH 1:40,000 (Biotrend 5G4MAB6C5). After three washes, membranes were incubated with horseradish peroxidase labeled anti-rabbit or anti-mouse IgG 1:10,000 for 1 h at room temperature (GE Healthcare NA934 & NXA931), washed three times and developed with chemiluminescent reagent (Immobilon Western Chemiluminescent HRP substrate, Millipore). The intensity of the signal was collected using a Kodak Imaging Station 4000R. Band densitometry analysis was performed using Carestream MI Software V.5.0.3. Data are reported as normalized change compared to sham.

Computational modeling of local Ca²⁺ release during heterogeneous CRU spacing

Local CRU Ca²⁺ sparks and cell-wide Ca²⁺ transients were investigated using the mathematical model of Williams et al. 2011,12 which includes spatial nanodomain determinants of individual CRU organization and a realistic number of 20,000 release sites. The whole-cell model is fully stochastic including Ca²⁺ spark behavior, and reproduces the systolic [Ca²⁺]i transient
throughout a propagated action potential activating L-type Ca\(^{2+}\) channels (LCC) at the surface membrane and at TTs. The LCC current elevates \([\text{Ca}^{2+}]\), locally in the "subspace" compartment between the TT and terminal SR membrane, which is greatly amplified when RyR2 clusters are activated by locally elevated subspace \([\text{Ca}^{2+}]\), as occurs during CICR. The recently developed mathematical model examines how local subspace \([\text{Ca}^{2+}]\), signaling changes between control and HF conditions by implementing previously observed (published) changes in ion transport proteins: fast and slow K\(^+\) currents (I\(_{\text{lo}}\) and I\(_{\text{k}}\)) were reduced by 30\%; NCX protein expression was increased by 100\%;\(^{13}\) SR Ca\(^{2+}\) ATPase (SERCA2a) protein was decreased by 30\%;\(^{14}\) and RyR2 Ca\(^{2+}\) sensitivity was increased by 50\% to mimic increased activity from chronic hyperphosphorylation.\(^1\) In addition, "orphaning" of RyR2 clusters during heart failure during heterogeneous changes of individual TT structures (as reported by this study) was modeled as a 30-fold increase in subspace volume for only 25\% of CRUs and as a reduction of the transversal TT component leading to a 20\% decreased total membrane capacitance. The increased subspace volume of diseased CRU release sites are the equivalent of moving individual TT membranes 300 nm away from associated CRU release sites.

**Statistics**

Data are presented as mean ± SEM unless indicated otherwise. Differences between groups were tested for statistical significance using the unpaired 2-tailed or paired Student’s t-test as appropriate. The nonparametric Mann-Whitney test was used to determine significant differences in median TT radius distributions. P values less than 0.05 were considered significant.
B. Supplemental Tables

Online Table I

T-tubule dimensions at subsurface locations in untreated control myocytes imaged by confocal versus STED mode.

<table>
<thead>
<tr>
<th>Imaging mode</th>
<th>Diameter X (nm)</th>
<th>Diameter Y (nm)</th>
<th>Area (1000 nm²)</th>
<th>Circumference (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confocal</td>
<td>273.1 ± 2.7</td>
<td>266.1 ± 2.1</td>
<td>57.2 ± 0.7</td>
<td>945.3 ± 6.7</td>
</tr>
<tr>
<td>STED</td>
<td>206.0 ± 3.5 *</td>
<td>194.6 ± 2.7 *</td>
<td>31.8 ± 0.7 *</td>
<td>708.6 ± 9.8 *</td>
</tr>
</tbody>
</table>

TT cross-sections at subsurface locations were directly compared by confocal versus STED mode less than 3 μm from the surface membrane. TT diameters represent FWHM measured by automated 2D Gauss analysis corresponding to X (longitudinal) and Y (transverse) orientations (as given by Figure 1A). Circumference was determined by automated contour analysis (see Online methods). Data represent paired measurements; *p<0.001 vs. confocal imaging; mean ± SEM from 182 TT cross-sections and 55 cells.
Online Table II

T-tubule dimensions at subsurface locations during HF development.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Diameter X (nm)</th>
<th>Diameter Y (nm)</th>
<th>Area (1000 nm²)</th>
<th>Circumference (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham a</td>
<td>201.0 ± 2.0</td>
<td>190.5 ± 1.8</td>
<td>30.3 ± 0.4</td>
<td>656.0 ± 5.6</td>
</tr>
<tr>
<td>4pMI b</td>
<td>199.5 ± 2.7 n.s.</td>
<td>190.3 ± 2.6 n.s.</td>
<td>29.8 ± 0.5 n.s.</td>
<td>649.4 ± 7.5 n.s.</td>
</tr>
<tr>
<td>8pMI c</td>
<td>214.7 ± 3.3 *†</td>
<td>205.7 ± 3.3 *†</td>
<td>35.0 ± 0.8 *†</td>
<td>701.0 ± 10.4 *†</td>
</tr>
</tbody>
</table>

TT cross-sections at subsurface locations were imaged by STED less than 3 μm from the surface membrane from randomly selected cells of the indicated treatment groups. TT diameters represent FWHM measured by automated 2D Gauss analysis corresponding to X (longitudinal) and Y (transverse) orientations. Circumference was determined by automated contour analysis. Data represent unpaired measurements; *p<0.05 vs. sham; †p<0.05 vs. 4 weeks post-MI; n.s., not significant; mean ± SEM from the following number of TT cross-sections/cells per treatment group: a sham 683/92, b 4 weeks post-MI (4pMI) 343/61, and c 8 weeks post-MI (8pMI) 253/48.
Online Table III

Length of the T-tubule network, its components, and post-MI changes.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total Network Length (μm/μm²)</th>
<th>Transversal Component Length (μm/μm²)</th>
<th>Longitudinal Component Length (μm/μm²)</th>
<th>Oblique Component Length (μm/μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham a</td>
<td>0.38 ± 0.02</td>
<td>0.020 ± 0.001</td>
<td>0.024 ± 0.002</td>
<td>0.018 ± 0.002</td>
</tr>
<tr>
<td>4pMI b</td>
<td>0.52 ± 0.04 *</td>
<td>0.020 ± 0.002 n.s.</td>
<td>0.045 ± 0.006 *</td>
<td>0.026 ± 0.002 *</td>
</tr>
<tr>
<td>8pMI c</td>
<td>0.50 ± 0.03 *</td>
<td>0.021 ± 0.003 n.s.</td>
<td>0.032 ± 0.003 †</td>
<td>0.028 ± 0.002 *</td>
</tr>
</tbody>
</table>

Length normalized to area of the T-tubule network and its longitudinal, transversal, and oblique components. We report the total length normalized to analyzed ROI area for each component (μm/μm²) as well as changes after myocardial infarction. Longitudinal (0°) and transversal (90°) T-tubule components represent ± 3° large bins. Oblique components represent the sum of +45° ± 3° and -45 ± 3° bins. Data represent unpaired measurements; *p<0.01 vs. sham; †p<0.05 vs. 4 weeks post-MI; n.s., not significant; mean ± SEM from the following number of cells per treatment group: a sham 23, b 4 weeks post-MI (4pMI) 19, and c 8 weeks post-MI (8pMI) 19.
Online Table IV

T-tubule changes in ventricular myocytes reported by experimental studies or investigation of patient samples

A. Experimental studies of heart disease models and associated T-tubule changes (in chronological order)

<table>
<thead>
<tr>
<th>Disease model</th>
<th>Time point studied</th>
<th>T-tubule changes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pacing-induced canine HF</td>
<td>~5 weeks after pacing</td>
<td>Regional loss, reduced density</td>
<td>He, 2001</td>
</tr>
<tr>
<td>Pacing-induced canine HF</td>
<td>~6 weeks after pacing</td>
<td>Regional loss</td>
<td>Balijepalli, 2003</td>
</tr>
<tr>
<td>Spontaneously hypertensive rat with congestive HF</td>
<td>at 19.2 months age</td>
<td>Reduced transversal network component, chaotic network reorganization</td>
<td>Song, 2006</td>
</tr>
<tr>
<td>Mouse post-MI HF</td>
<td>1 week or 3 weeks after MI</td>
<td>Regional disorganization; 1 week “somewhat”; 3 weeks: “marked”</td>
<td>Louch, 2006</td>
</tr>
<tr>
<td>Pig post-MI HF</td>
<td>6 weeks after MI</td>
<td>Regional loss, reduced density</td>
<td>Heinzel, 2008</td>
</tr>
<tr>
<td>Rat post-MI end-stage HF</td>
<td>16 weeks after MI</td>
<td>Regional loss, reduced density</td>
<td>Lyon, 2009</td>
</tr>
<tr>
<td>Rat heterotopic abdominal heart transplantation</td>
<td>4 weeks of cardiac unloading</td>
<td>Network reorganization, loss of surface connections</td>
<td>Ibrahim, 2010</td>
</tr>
<tr>
<td>Rat aortic banding hypertrophy and HF</td>
<td>9.2 weeks hypertrophy; or 9.9 weeks HF</td>
<td>Regional loss, different degrees of TT loss from “discrete” to “dramatic”</td>
<td>Wei, 2010</td>
</tr>
<tr>
<td>Inducible cardiac-specific knockdown of Junctophilin-2</td>
<td>1 week after knockdown induction, adult heart</td>
<td>Regional loss, increased CRU spacing and loss of CRU junctions</td>
<td>van Ort, 2011</td>
</tr>
<tr>
<td>PI3K p110α/β double knockout</td>
<td>5 weeks age</td>
<td>Regional loss</td>
<td>Wu, 2011</td>
</tr>
<tr>
<td>Rat post-MI HF</td>
<td>4 weeks post-MI</td>
<td>Reduced TT density</td>
<td>Kemi, 2011</td>
</tr>
<tr>
<td>Canine dyssynchronous HF</td>
<td>6 weeks after intervention</td>
<td>Sparse at cell center; longitudinal increase</td>
<td>Sachse, 2012</td>
</tr>
</tbody>
</table>

B. Studies of patient samples from diseased hearts and associated T-tubule changes (in chronological order)

<table>
<thead>
<tr>
<th>Disease studied</th>
<th>Sample studied</th>
<th>T-tubule changes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Different HCM samples</td>
<td>Fixed biopsy sections</td>
<td>Loss and dilation of TTs</td>
<td>Maron, 1975</td>
</tr>
<tr>
<td>End-stage DCM</td>
<td>Fixed tissue sections</td>
<td>Proliferation and dilation</td>
<td>Schaper, 1991</td>
</tr>
<tr>
<td>End-stage DCM</td>
<td>Fixed tissue cryosections</td>
<td>Regional loss and dilations</td>
<td>Kostin, 1998</td>
</tr>
<tr>
<td>End-stage DCM, ICM</td>
<td>Fixed tissue sections</td>
<td>Increase in size and longitudinal elements</td>
<td>Kaprielian, 2000</td>
</tr>
<tr>
<td>End-stage DCM, ICM</td>
<td>Isolated living myocytes</td>
<td>Normal density, occasional dilation</td>
<td>Louch, 2004</td>
</tr>
<tr>
<td>End-stage tachycardia HF</td>
<td>Fixed tissue sections</td>
<td>Reorganization, increase oblique TTs</td>
<td>Cannell, 2006</td>
</tr>
<tr>
<td>End-stage DCM, HCM, ICM</td>
<td>Isolated living myocytes</td>
<td>Regional TT loss, reduced TT density</td>
<td>Lyon, 2009</td>
</tr>
<tr>
<td>End-stage DCM</td>
<td>Fixed tissue sections</td>
<td>Loss transverse TTs, increase oblique TTs</td>
<td>Crossmann, 2011</td>
</tr>
</tbody>
</table>

Abbreviations: DCM, Dilated Cardiomyopathy; HCM, Hypertrophic Cardiomyopathy; ICM, Ischemic Cardiomyopathy.
C. Online Figures

Online Figure I

**Imaging principles of STED nanoscopy.**

**A**, Using an inverted microscope (Leica) custom-modified for beam scanning and STED microscopy, the STED beam is switched ON/OFF by a shutter for super-resolution versus confocal imaging, respectively. APD, avalanche photo diode; DM, dichroic mirror; Exc, excitation laser; SMF, single mode fiber; Trigger, synchronizes STED and excitation laser pulses; PP, phase plate; RSM, resonant scanning mirror.

**B**, Nanobead (20 nm) measurements document significantly reduced focal spot size below the diffraction barrier for STED. **Left:** xy intensity projection of individual data from n nanobeads (given by tables) showing lateral signal spread for confocal and STED modes, and average point-spread-functions. **Right:** xz intensity projection for confocal and STED nanobead measurements, and average point-spread-functions. **Bottom:** Tables summarizing average FWHM for confocal and STED modes, and numbers of measured nanobeads. Conf, confocal; scale 200 nm.

<table>
<thead>
<tr>
<th>Confocal</th>
<th>STED</th>
</tr>
</thead>
<tbody>
<tr>
<td>FWHM Mean ± SD</td>
<td>n</td>
</tr>
<tr>
<td>x</td>
<td>191 ± 11</td>
</tr>
<tr>
<td>y</td>
<td>203 ± 21</td>
</tr>
<tr>
<td>z</td>
<td>455 ± 21</td>
</tr>
</tbody>
</table>
Online Figure II

STED imaging of TT cross-sections at “submembrane” locations less than 3 μm from the cell surface of living control myocytes.

A, Histograms of the following TT cross-section dimensions: diameter X, left; diameter Y, right each from confocal (upper) versus STED (lower) data imaged less than 3 μm from myocyte surface membrane. Diameters represent full width at half maximum (FWHM) each for longitudinal (X) and transversal (Y) directions, determined by fitting a 2D Gaussian function to the fluorescence intensity distribution (see Online methods). Vertical red line marks 220 nm, the confocal resolution limit. B, Same TT cross-sections (n = 182) as in (A) were analyzed by automated contour algorithm using a 50% intensity threshold (see Online methods). Contour data are presented as 2D probability histogram each for confocal and STED mode documenting differences in distribution. Contours are centered by origin (indicated by ‘+’). Colors indicate pixel probabilities as given by look-up-table. C, STED versus confocal imaging near the surface membrane results in different median distributions of radius sizes (grey dashed lines; *p<0.05, non-parametric Mann-Whitney test). Radius sizes were determined from individual TT cross-sections by contour analysis (see methods). In addition, Gaussian fitting confirmed a left-shifted and wider distribution of radius sizes for STED (red line) versus confocal (black line) mode confirming faithful detection of individual TT structures. Mean data are summarized in Online Table I.
In vivo cardiac phenotype and progressive remodeling after myocardial infarction.

A, Heart weight normalized to body weight was significantly increased at 4pMI and 8pMI. B, Echocardiographic measurements showed a significant decrease in ejection fraction at 4pMI and 8pMI. C, Left ventricular end-diastolic cross-section area (Area, d) was significantly increased at 4pMI and 8pMI. Bar graphs represent unpaired measurements; *p<0.05 vs. sham; †p<0.05 versus 4pMI; means ± SEM; animal numbers are indicated in (A) and reflect only hearts used for isolated cell analysis.
Myocyte dimensions increase progressively during HF development following myocardial infarction. Top: Example cell from a sham heart. Myocyte dimensions were determined by automated surface/contour analysis (red line) from randomly selected, laminin plated, and fixed cells of different treatment groups by transmitted light imaging. We used freely available ImageJ software for analysis (http://rsbweb.nih.gov/ij). Bar graphs document progressive increase of cell dimensions by area, length, width, and ratio length:width at 4pMI and 8pMI versus sham cells. Numbers of cells are indicated in first bar graph. Symbols: *p<0.05 vs. sham; †p<0.05 vs. 4pMI; n.s., not significant.
Online Figure V

STED imaging of TT cross-sections at "subsurface" locations less than 3 μm from the cell surface during HF development.

A, Confocal versus STED image examples of the same enlarged subsurface TT cross-section at 8pMI. Scale 200 nm.


C, Longitudinal (X) and transversal (Y) diameters at subsurface locations were determined as FWHM from TT cross-sections (see methods). Bar graphs summarize mean TT diameters X and Y, and cross-section area; right: percent change in TT cross-section dimensions normalized to sham. *p<0.05 versus sham; †p<0.05 versus 4pMI; n.s., not significant.

D, 2D probability histograms of contour data from subsurface TT cross-sections of the indicated treatment groups (same TT cross-sections as in C). TT cross-sections were analyzed by automated contour algorithm (see methods). Colors represent highest (white) versus lowest (black) contour pixel probabilities as indicated by look-up-table.

E, Difference integrals were calculated from averaged data of individual TT radius sizes and presented as difference between the indicated treatment groups. Compared to sham, a decrease of small versus an increase of large TT radius sizes occurred during HF development. However, at 4pMI the changes are less pronounced at subsurface compared to deep intracellular locations (see Figure 3F).

F, Median radius size distributions calculated from individual subsurface TT contours of sham and 8pMI cells (grey dashed lines). The 8pMI distribution is significantly right-shifted (*p<0.05). Furthermore, Gaussian fitting of the 8pMI data (red line) confirmed a rightward shift toward increased radius sizes at 8pMI. However, the changes were less pronounced at subsurface as compared to deep intracellular locations (see Figure 3G). Mean data are summarized in Online Table II.
Optical sectioning and surface topography of TT substructures.

A, Selected examples of a live cell STED image stack showing TT cross-sections in different imaging depth (z); images were processed according to the PSF given by online figure IB. Numbers correspond with indicated z positions in (B), marked by arrowheads.

B, 3D side view (90° rotation) of entire TT image stack from the myocyte surface (top) toward the cell center (19 sections every 200 nm). Note membrane evaginations of selected TT cross-sections (planes 1, and 11 and higher).

C, 3D surface topography further rotated to highlight lower TT section containing membrane evaginations.

D, Graph showing fold deviation (> 1) from an ideal circular TT cross-section symmetry, determined as the ratio of longitudinal over transversal diameter length by manual contour analysis. Note that TT cross-sections deviating from a circular symmetry (up to 4-fold) correspond with imaging sections containing TT membrane evaginations.

E, Top view from the cell surface showing two reconstructed transversal TTs at neighboring sarcomere striations from a sham heart; and F, at 8pMI two neighboring transversal TTs continuously connected through a longitudinal TT element and super-enlarged cross-sections resulting in a complex, abnormal membrane morphology (see also Figure 3B, Figure 4 and results section for analysis of super-enlarged TT cross-sections). Sham and 8pMI surface reconstructions represent 7 image planes every 300 nm using the indicated 3D orientation; scale 1 μm.
Analysis of TT associated protein clusters by confocal imaging of immunostained LV cardiomyocytes from post-MI or sham hearts.

Using Fast Fourier transformation, the striation associated periodic signal amplitude represented by the first spatial frequency (at 0.55 \( \mu m^{-1} \)) was analyzed each for the indicated Cav3, RyR2, and Jph2 cluster changes post-MI. Bar graphs summarize the power amplitude at the spatial frequency of 0.55 \( \mu m^{-1} \) (indicated by triangle) of sham versus post-MI groups as indicated. Figure 5A shows corresponding representative co-immunofluorescence images.

Cav3: The Cav3 power spectrum at 4pMI was upward shifted consistent with a more heterogeneous distribution while protein expression is significantly increased. The peak amplitude at the spatial frequency of 0.55 \( \mu m^{-1} \) indicated by triangle was significantly decreased indicating a loss of striation-associated Cav3 clusters.

RyR2: The RyR2 power spectrum was changed at 4pMI compared to sham showing a significantly reduced amplitude at the spatial frequency of 0.55 \( \mu m^{-1} \) indicated by triangle. The decreased peak signal together with increased signal between periodic peaks suggests a loss of RyR2 clusters at striations post-MI, possibly due to spatial reorganization since RyR2 protein expression was unchanged.

Jph2: The power versus frequency function was downward shifted at 8pMI, while protein expression was significantly decreased post-MI. In addition, the spatial frequency at 0.55 \( \mu m^{-1} \) indicated by triangle was significantly decreased consistent with a loss of Jph2 clusters at striations.

*, p<0.05.
Direct comparison of STED and confocal measurements of TT subresolution structures to estimate the detection sensitivity for nanometric post-MI changes. All measurements were performed using the same optical microscopy system as described in Online Figure I-A.

**Top:** TT cross-sections dimensions were analyzed by 2D FWHM algorithm for the same sample TT samples of the indicated treatment groups (numbers as given by Table 2). STED measured significantly smaller TT cross-section dimensions in sham cardiomyocytes confirming the results shown in Figure 2 from untreated control cells. Despite significantly smaller TT dimensions, only STED detects a highly significant increase of the cross-section area at 4pMI, the early HF time point, a relatively subtle disease change clearly not resolved by confocal measurements. While confocal measurements detect a significant mean area increase at 8pMI (+7%), the relative increase measured versus sham is highly significantly larger for STED measurements (+22%; p<10^-40 versus confocal group at 8pMI).

**Bottom:** Triple junctions or TT bifurcations were analyzed by direct automated image quantification (see methods) as an indicator of network complexity. The normalized data show a dramatic increase of the number of triple junctions relative to sham at 8pMI, yet this progressive post-MI increase is only detected by STED, whereas the confocal data shows a tendency for a relative decrease at 8pMI. For image examples of TT network complexity please refer to Figures 3C and 4A.

*, p<0.05; **, p<0.001; ***, p<0.0001 each versus corresponding sham group; only changes versus sham are indicated for clarity. STED: hatched bars; confocal: open bars.
D. Supplemental References


18. Lyon AR, MacLeod KT, Zhang Y, Garcia E, Kanda GK, Lab MJ, Korchev YE, Harding SE, Gorelik J. Loss of T-tubules and other changes to surface topography in ventricular


