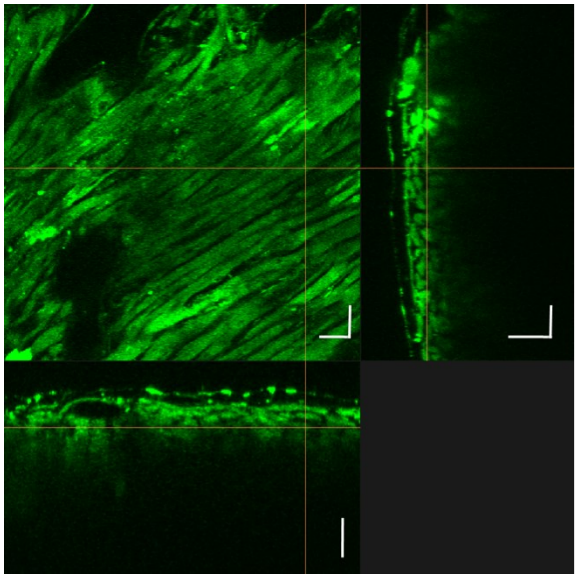
# Supplemental Data

## *Supplemental Methods*

The preparation of the heart and Langendorff perfusion has been described before [1]. Briefly, the heart was placed in a perfusion chamber with a glass bottom for inverse microscopes and two AgCl pellet electrodes were placed in the bath in close proximity of the heart to record the EKG signal during the course of an experiment. This allows monitoring the viability of the heart and the remaining intrinsic heart rate. Platinum stimulation electrodes were positioned at the base of the heart to ensure that the stimulus is delivered to the same region of the heart in relation to the region where $Ca^{2+}$ imaging was performed. For experiments performed with an upright microscope, the heart was positioned in a larger perfusion chamber, giving enough space to the objective. In this setting the heart needed to be positioned in the chamber with a smaller angle exposing the left ventricular epicardium towards the objective. 2D time series of hearts loaded with Fluo8-AM were acquired with a 2-photon excitation microscope (Nikon A1MP, Nikon Instruments Inc., Melville, NY, USA). The fluophore was excited with an excitation wavelength of 930 nm and emission was collected at >530nm. The scan speed of 50 fps was achieved with a resonant scanner with a spatial resolution of 0.9 m/pixel.

## *Supplemental Results*

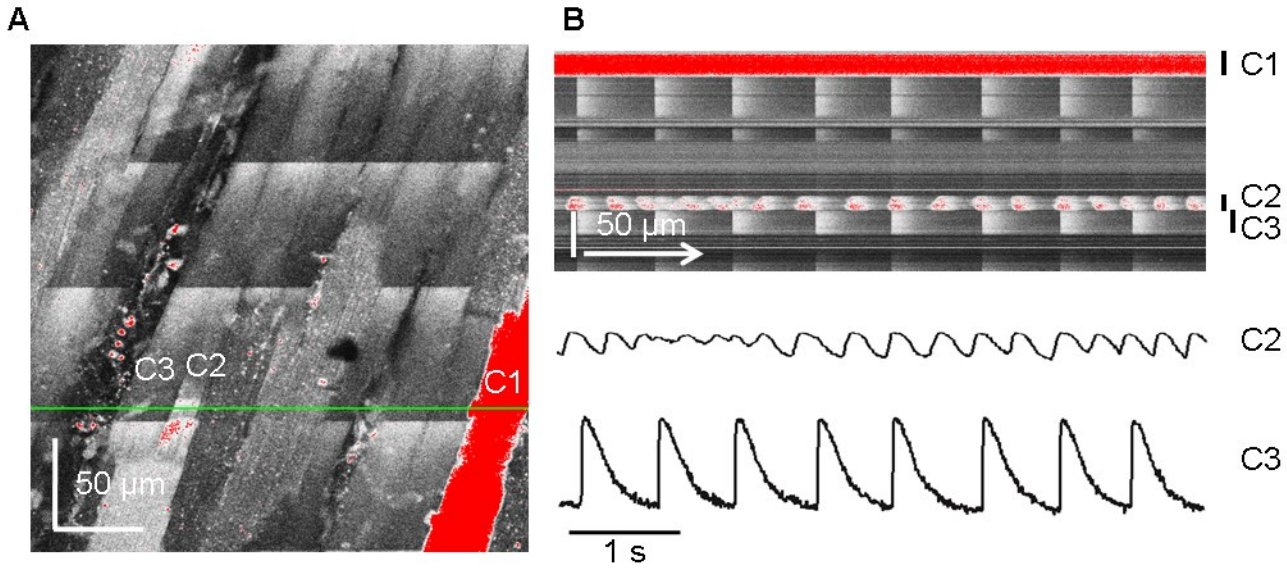
The penetration depth of the confocal microscope into the cardiac tissue was about 17 µm, which means only the first cardiac layer of the epicardial surface could be observed. While a multi-photon excitation laser can penetrate deeper into the tissue compared to the single photon excitation laser, the penetration depth into cardiac tissue was still limited to about 36 µm, adding just 1 additional layer of cells that can be observed. Z-stacks reveal that the myocardial layer is covered by an epithelial cell layer that is also labeled with the Fluo8-AM (supp. Fig. 1).



*Supplemental Figure 1: z-stack of the epicardial layer of the intact heart*

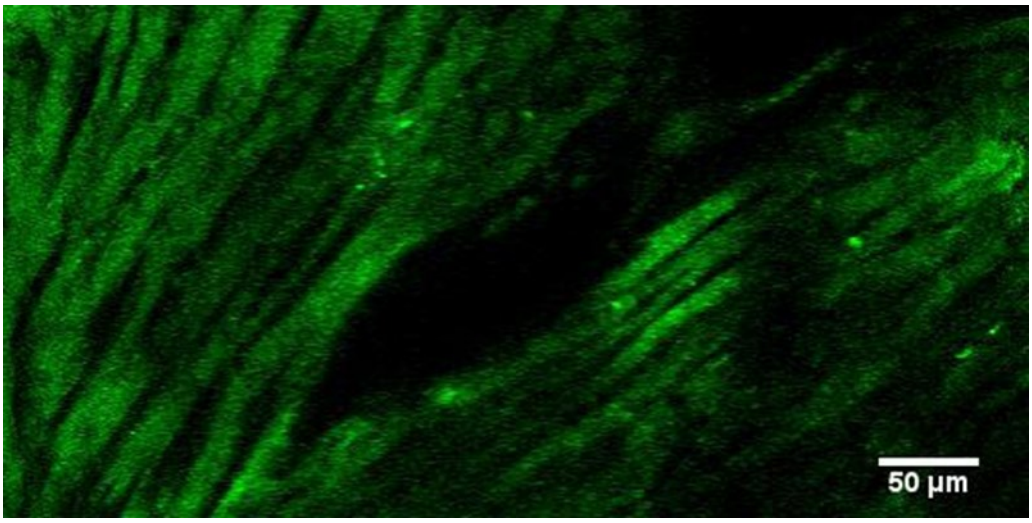
Z-stack of the epicardial layer of a mouse heart loaded with Fluo8-AM acquired with two-photon microscopy. In the center the focal plane is visible with two lines crossing at one point. The two lines indicate the location of the x/z and y/z images below and besides the x/y plane with the lines indicating the position of the focal plane displayed in the main frame; the scale bars indicate 50 µm. A thin layer above the cardiac myocytes can be seen, probably resembling a layer of endothelial cells surrounding the heart. The two-photon technique can acquire data up to a penetration depth of 36 µm compared to 17 µm achieved by a conventional confocal microscope.

In our experiments we found that $Ca^{2+}$ waves occasionally occur during high frequency stimulation or after cessation of stimulation during phases of high $Ca^{2+}$ load in cardiac myocytes. In concordance with other reports [2-4], we did not observe the spread of waves from cell to cell in intact tissue. In fact, even severely $Ca^{2+}$ overloaded cells did not affect $[Ca^{2+}]_i$ in their neighbors during excitation contraction coupling and single cells with $Ca^{2+}$ waves did not alter the ECC pattern of the neighboring cells. The example shown in suppl. Fig. 2 suggests that cells with adverse behavior are being isolated from their neighbors in normally coupled tissue. This potentially poses a mechanism to prevent altered $Ca^{2+}$ cycling in single cells to spread and cause functional irregularities on the organ level.




*Supplemental Figure 2: line-scan images of overloaded and spontaneously active cells within the intact tissue.*
The snapshot in (A) shows the 2D view of the surface of a heart loaded with Fluo8-AM taken with a conventional confocal microscope. The lighter areas indicate high Ca concentrations due to the acquisition time of the image (3s) 3 transients occurred during the acquisition and the field of view had a size of 256 x 256 µm (bar graphs = 50 µm). The green line indicates the position of the line scan that is depicted in (B). 3 cells are highlighted: C1 is an overloaded cell that no longer reacts to any stimulus; C2 is a spontaneously active cell with a high frequency of $Ca^{2+}$ waves neighboring C3, a cell regularly exhibiting Ca transients in synchrony with the other cells in the field of view. The traces of the $Ca^{2+}$ signals of the adjacent cells C2 and C3 confirm that the two cells seem to be disconnected from each other.

The 2D time series of the epicardial surface of a mouse heart loaded with Fluo8-AM supports the findings from confocal microscopic studies where $Ca^{2+}$ waves stay confined within one cell. The movie shows the simultaneous Ca rise in the neighboring cells within the field of view representative for synchronous ECC in intact tissue with normally coupled cells. The cell indicated by the arrow shows spontaneous activity independent of the other cells. The stimulus that assures a synchronous ECC in the heart does not seem to reach this cell and the adverse $Ca^{2+}$ handling of this single cell does not affect its neighbors in the field of view. This example also supports the notion that $Ca^{2+}$ waves stay confined within one cell and do not spread to its adjacent neighbors. These findings support our findings that intercellular communication plays a pivotal role in maintaining stable $Ca^{2+}$ cycling and contractility in the heart.




*Movie: $Ca^{2+}$ transients in the beating heart.*
The spontaneously beating mouse heart was loaded with Fluo8-AM and the Ca transients were recorded with a 2 photon microscope; the acquisition speed was 50 fps. The cells display synchronous $Ca^{2+}$ cycling within the field of view. A single cell in the lower right of the field of view displayed spontaneous $Ca^{2+}$ release in the form $Ca^{2+}$ waves. These waves were independent of the global stimulus and did not affect the $Ca^{2+}$ handling of the neighboring cells.

## *Supplemental Literature*

[1] Hammer K, Lipp P, Kaestner L. Multi-beam 2-photon imaging of fast $Ca^{2+}$ signals in Langendorff mice heart. Cold Spring Harb Protoc. 2014.

[2] Wasserstrom JA, Shiferaw Y, Chen W, Ramakrishna S, Patel H, Kelly JE, et al. Variability in timing of spontaneous calcium release in the intact rat heart is determined by the time course of sarcoplasmic reticulum calcium load. Circulation research. 2010;107:1117-26.

[3] Kaneko T, Tanaka H, Oyamada M, Kawata S, Takamatsu T. Three distinct types of $Ca^{2+}$ waves in Langendorff-perfused rat heart revealed by real-time confocal microscopy. Circulation research. 2000;86:1093-9.

[4] Baader AP, Buchler L, Bircher-Lehmann L, Kleber AG. Real time, confocal imaging of $Ca^{2+}$ waves in arterially perfused rat hearts. Cardiovascular research. 2002;53:105-15.