

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Heart Preparation

Female New Zealand White rabbits (1.35 ± 0.35 kg) were euthanized by ear vein injection of 140 mg/kg pentobarbital, as approved by the local ethical review committee and in accordance with the UK Home Office *Animals (Scientific Procedures) Act* of 1986. After thoracotomy, the heart was swiftly excised and placed in Krebs-Henseleit solution (containing [in mM]: 120 NaCl; 4.7 KCl; 24 NaHCO₃; 1.4 NaH₂PO₄; 1.0 MgCl₂, 1.8 CaCl₂; 10 Glucose; osmolality: 301 ± 4 mOsm/kg; pH: 7.42 ± 0.04) bubbled with carbogen (95% O₂, 5% CO₂). The heart was connected rapidly ($2:00 \pm 0:33$ min from excision) bubble-free to a custom Langendorff apparatus by aortic cannulation and perfused with Krebs-Henseleit solution at 15 mL/min (temperature: $37.0 \pm 0.5^\circ\text{C}$). Perfusion pressure was monitored with a transducer (TSD104A; Biopac Systems Inc., Goleta, CA), and temperature with a fast-response thermistor (TSD202A; Biopac Systems Inc.) positioned in the aortic cannula *via* a three-way stopcock. An incision into the proximal pulmonary artery allowed coronary effluent to exit the right ventricle (RV). Remaining extra-cardiac tissue (lungs, thymus, pericardium, vessels) was removed. For mechanical support during epicardial mechanical stimulation, the heart was positioned into an individually pre-molded Parafilm (Bemis Company Inc., Oshkosh, WI) cradle with black backing, and the entire perfusion system was angled at 45° , with the ventricle of interest facing upwards, to allow surface-perpendicular mechanical contact during optical measurements. The exposed epicardial surface was superfused with warm Krebs-Henseleit solution at a rate of 1 mL/min.

A 4 - 5 mm incision was made either in the mid-left or mid-right atrial auriculum, depending on the ventricle being mechanically stimulated. A short piece of 18G intravenous cannula was passed through the incision and across the mitral or tricuspid orifice into the left ventricle (LV) or RV and pushed transmurally through the apex to allow outflow of coronary effluent. A custom-made hydrostatically pre-stained deflated polyethylene balloon, fitted on a 10 mm piece of manometer line (2 mm inner diameter) filled with degassed water and connected to a three-way stopcock, was inserted into the ventricle *via* the auricular incision. The balloon tip was secured at the ventricular apex by a 3-0 silk suture through the apical cannula. The atrium was tied to the manometer line by a silk ligature to secure the base of the balloon inside the ventricle. A 500 μL glass syringe, filled with degassed water, was connected to the balloon stopcock for active adjustment of balloon volume. Intra-ventricular pressure

was monitored with a transducer (TSD104A; Biopac Systems Inc.) connected to the balloon stopcock. The balloon, syringe, pressure transducer, and connections were kept air-free, and the distance between the syringe piston and the balloon inflow was minimized, to prevent damping or resonance of the pressure signal during rapid volume injection and removal. A surface ECG was measured using two spring-loaded monopolar Ag/AgCl pellet electrodes (PY2 73-0200; Harvard Apparatus, Holliston, MA), one contacting the right atrium and the other the LV apex. Temperature, perfusion pressure, and ECG sensors were interfaced with a data acquisition system (MP150; Biopac Systems Inc.) and data collected at 2 kHz. After a 15 min equilibration period, the intra-ventricular balloon was filled with degassed water using the attached syringe until ventricular diastolic pressure was between 0 and 5 mmHg. The experimental setup can be seen in Supplemental Fig. S1.

Voltage Optical Mapping

Following preparation, hearts were loaded with a voltage-sensitive dye (di-4-ANBDQPPQ; acquired from the University of Connecticut Health Center, Farmington, CT)¹ by direct injection into the aortic cannula (20 μ L bolus of 35.1 mM solution in medical grade ethanol, delivered in 0.4 μ L increments over 2 min, *i.e.*, diluted in 30 mL of perfusate to 23.4 μ M). An excitation-contraction uncoupler (10 μ M (\pm)-Blebbistatin; Abcam Inc., Cambridge, United Kingdom) was added to 600 mL perfusate, to eliminate contraction-induced mapping-artifacts in optical recordings.² The perfusate was recirculated through an 11 μ m pore nylon net filter (NY11; EMD Millipore, Billerica, MA) for the remainder of the experiment.

After a 30 min period to allow blebbistatin to act, optical mapping was performed using a previously described custom optical mapping system.³ Fluorescence was excited by red light-emitting diodes (CBT-90-R; Luminus Devices Inc., Billerica, MA) using a band-pass filter (D640/20X; Chroma Technology Corp., Bellows Falls, VT). Emission was collected through a 50 mm high-speed lens (DO-5095; Navitar, Rochester, NY) and recorded at 511 frames per second (fps) by a 128 \times 128 pixel, 16-bit electron multiplying charge coupled device camera (Cascade:128+; Photometrics, Tucson, AZ) using a long-pass filter (HQ690LP Chroma Technology Corp.). The camera was controlled and signals were acquired using MultiRecorder (kindly shared by Stefan Luther and Johannes Schröder-Schetelig, Max Planck Institute for Dynamics and Self-Organisation, Göttingen, Germany; <http://www.bmp.ds.mpg.de/multirecorder.html>).

Mechanical Stimulation

Local mechanical stimuli were applied at various sites of the ventricular epicardium using the Soft Tissue Impact Characterisation Kit.⁴ The device comprises a low-friction rail and carriage system, with the carriage guiding a smooth-edged cylindrical contact probe to the target. Prior to, and during, tissue contact, carriage position is monitored using an optical grating passing through two stationary photodiodes, spaced at half the line-width of the optical grating (71 $\mu\text{m}/\text{line}$). Upon complete deceleration of the probe by tissue contact, a retractor arm immediately lifts it back up the rail to prevent secondary mechanical interactions between probe and heart. The rail was angled at 90° to the epicardial stimulation site on the ventricular surface. The combined carriage and probe mass was 2.89 g for both contact surface areas (3.1 mm^2 or 28.3 mm^2) tested. Local mechanical stimulation characteristics were altered by varying carriage drop height and/or probe contact area. A typical stimulation is illustrated in Fig. 1 and Movie S1.

Intra-ventricular pressure surges in the absence of local epicardial stimulation were applied by rapid changes in intra-ventricular balloon volume, involving active loading *and* active unloading. This was accomplished by swift forward and reverse motion of a computer-controlled linear DC-servomotor (LM 1247-02-01; Faulhaber Mini Motor SA, Croglio, Switzerland) coupled to the balloon syringe. Volume displacement was controlled and verified by a motion controller (MCLM 2006 S; Faulhaber Mini Motor SA) using custom programs developed in Motion Manager (Faulhaber Mini Motor SA).

Local mechanical stimulation and pressure-surge timing relative to the cardiac cycle (coupling interval) were controlled by calibrating carriage drop or servomotor delay times, respectively, triggering interventions from the peak of the ECG R-wave using custom-designed electronics and programs developed in MATLAB (MathWorks, Natick, MA). Carriage position and intra-ventricular pressure were acquired with a Biopac data acquisition system at 100 kHz. To verify that mechanical stimuli were sub-contusional, tissue integrity was assessed by analysis of creatine kinase activity in coronary effluent (17296H CK-NAC Liquid; Alpha Laboratories Ltd., Eastleigh, United Kingdom) using a spectrophotometer (BioTech UV1101; Biochrom WPA, Cambridge, United Kingdom), which has been previously shown to reliably track tissue damage associated with contusional mechanical stimulation in rabbit isolated hearts.⁴

Experimental Protocols

Four experimental series were performed.

The first set of experiments tested the inducibility of PVE_M and VF with local mechanical stimulation ($n = 32$; all n -numbers relate to number of heart preparations included). Carriage drop height was set to

produce sub-contusional stimuli of ~ 0.5 mJ, using the smaller (3.1 mm^2) probe contact area as the standard intervention. While local mechanical stimulation timing was triggered from the preceding ECG R-wave, stimulation coupling interval was assessed as the time-difference between the peak of the T-wave and tissue contact, to account for inter- and intra-individual variability in R-T duration. Coupling interval was shortened from late-diastolic (150 ms after the peak of the T-wave) until either VF was induced or PVE_M ceased to be elicited (when stimulation timing entered the refractory period, usually around the peak of the T-wave). Coupling interval was then varied over ± 20 ms in 5 ms steps from this timing, with at least two mechanical stimuli applied at each near-critical coupling interval. Local mechanical stimulation was applied to various sites of the mid-LV free wall in all hearts, as well as at additional locations in some of the hearts: LV apex ($n = 4$); LV base ($n = 4$); LV / RV border ($n = 4$); mid-RV free wall (with an intraventricular balloon in the RV; $n = 11$).

The second set of experiments investigated determinants of PVE_M threshold and compared properties of mechanically- and electrically-induced ventricular excitation ($n = 7$). Local mechanical stimuli were applied to various sites of the mid-LV and mid-RV free wall using 3.1 mm^2 and 28.3 mm^2 probes in a randomized order during late diastole (at $\sim 75\%$ of the cycle length). Carriage drop height was reduced from a distance producing stimuli of ~ 0.5 mJ until PVE_M ceased to be elicited (*i.e.*, when stimulation strength was below threshold). Carriage drop height was varied three times around the identified threshold value, with two or more stimuli applied at each height. Targeting the center of the mechanically stimulated locations, hearts were electrically stimulated using a point ($100 \text{ }\mu\text{m}$ diameter) concentric bipolar stimulation electrode (SNE-100; Lohmann Research, Castrop-Rauxel, Germany) with 2 ms bipolar pulses at $1.5\times$ threshold voltage (usually ~ 3 V).

The third set of experiments assessed the potential role of SAC_{NS} and K_{ATP} channels in mechanically-induced electrical responses. Substances known to block SAC_{NS} *in vitro* (increasing concentrations of 50, 250, and 500 μM streptomycin, $n = 6$, or 500 nm GsMTx-4, $n = 5$) or a K_{ATP} channel blocker (increasing concentrations of 5 and 10 μM glibenclamide, $n = 6$) were added and recirculated. Local mechanical stimuli of ~ 0.5 mJ (associated with 100% PVE_M induction in control conditions) were applied at the mid-LV freewall using the 3.1 mm^2 probe during diastole before drug application and after 15 min of continuous perfusion to allow drug action.

The fourth set of experiments examined the electrophysiological effects of intra-ventricular pressure surges ($n = 6$). Rapid changes in LV balloon volume by 20 μL produced pressure dynamics that most closely mimicked those measured during ~ 0.5 mJ PVE_M-inducing epicardial mechanical stimulation,

and were applied during diastole and during the ECG T-wave. This was repeated with volume changes increased, in 22 μL steps, up to 130 μL , to explore the ability to elicit PVE_M, or changes in repolarization. Tissue integrity was evaluated by creatine kinase assay.

Data Analysis

For all epicardial stimuli, local excitation was confirmed by voltage optical mapping. A 50 Hz low-pass temporal filter was applied to all signals and patterns of: (a) activation time (moment of maximum rate of V_m change); (b) 90% repolarization time (moment of V_m restoration to 90% of resting values, measured from moment of earliest repolarization in the associated map); and (c) action potential duration at 90% repolarization (difference between 90% repolarization and activation time, APD₉₀) were assessed (with maps smoothed by a 3-point moving average spatial filter). In cases where mechanically-induced VF occurred, the phase of electrical activity ‘under the probe’ during the approach to tissue contact was deduced by supplementing observed activity outside the area obscured by the probe with corresponding data from the preceding sinus beat.

Mechanically- and electrically-induced excitation from the same mid-LV freewall location were compared by calculating: (a) maximum rate of V_m change (dF_n/dt_{max} , in nominal units of normalized fluorescence per ms) at a site immediately adjacent to the tissue excited by mechanical stimulation (represented by the 6 ms isochrone); (b) APD₉₀ and APD at 50% repolarization (APD₅₀) at the same site; and (c) max conduction velocity, defined as the time between activation at the same site and at a point in line with the direction of fastest conduction near the ventricular apex.

Local mechanical stimulation characteristics were calculated as follows (Fig. 1B):

(a) Pre-Contact Kinetic Energy = $\frac{\text{Total Mass} \times \text{Pre-Contact Velocity}^2}{2}$, where total mass is the combined mass of carriage and contact probe, and pre-contact velocity is the maximum velocity of the carriage, reached just prior to tissue contact, defined as the moment when jerk (the rate of change of acceleration) first deviates from zero;

(b) Extent of Local Tissue Deformation, defined as the change in carriage position from first tissue contact until the moment when it reverses direction, defined as the moment when probe velocity goes to zero before changing polarity (moving upwards);

(c) Mean Rate of Local Tissue Deformation = $\frac{\text{Extent of Local Tissue Deformation}}{\text{Duration of Local Tissue Deformation}}$, where the duration of local tissue deformation is the time from first tissue contact until the moment when carriage direction reverses;

(d) Peak Stimulation Force=Total Mass \times Maximum Negative Carriage Acceleration (deceleration);

(e) Mean Stress (Pressure under the Probe) = $\frac{\text{Mean Stimulation Force}}{\text{Probe Area}}$, where mean stimulation force is defined as the pre-contact kinetic energy divided by the extent of local tissue deformation upon complete probe deceleration.

To characterize effects of pharmacological agents and of the application of pressure surges, the inducibility of PVE_M, the associated refractory period, and patterns of 90% repolarization time were measured and compared. For pressure surges, the relationship between intra-ventricular volume and pressure amplitude, pressure rise time (from baseline to maximum), and pressure reduction time (from maximum to 90% of baseline) were determined by linear regression.

Statistics

Data analysis was performed using custom programs in Matlab (MathWorks). Values are presented as mean \pm standard deviation. For statistical tests, a *p*-value < 0.05 was taken to indicate statistically significant differences between means. Comparison of local mechanically- and electrically-induced excitation and the effects of glibenclamide application were assessed by Wilcoxon signed-rank test (as distribution normality cannot be assumed). Mechanical stimulation characteristics at PVE_M threshold were compared by two-way ANOVA with Tukey-Kramer *post-hoc* test. Linear correlation between the change in intra-ventricular balloon volume and pressure surge amplitude, rise-time, or fall-time was assessed by Pearson's correlation.

SUPPLEMENTAL REFERENCES

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SUPPLEMENTAL FIGURES

Figure S1. Photographic image of experimental setup. Langendorff-perfused isolated heart (rabbit), instrumented with spring-loaded Ag/AgCl pellet electrodes for measurement of surface ECG and an intra-ventricular polyethylene balloon coupled to a microliter syringe with linear servomotor for volume control and transducer for pressure monitoring. Hearts were positioned at 45° from horizontal in a pre-molded Parafilm cradle to allow epicardial mechanical stimulation, using the Soft Tissue Impact Characterisation Kit (STICK; oppositely angled to yield ventricular surface-perpendicular contact) during optical measurements of transmembrane potential by excitation of voltage-sensitive dye with red light-emitting diodes (LED) and collection of fluorescence using an electron multiplying charge coupled device camera (EMCCD). For detail on the imaging method, see Lee *et al.*³

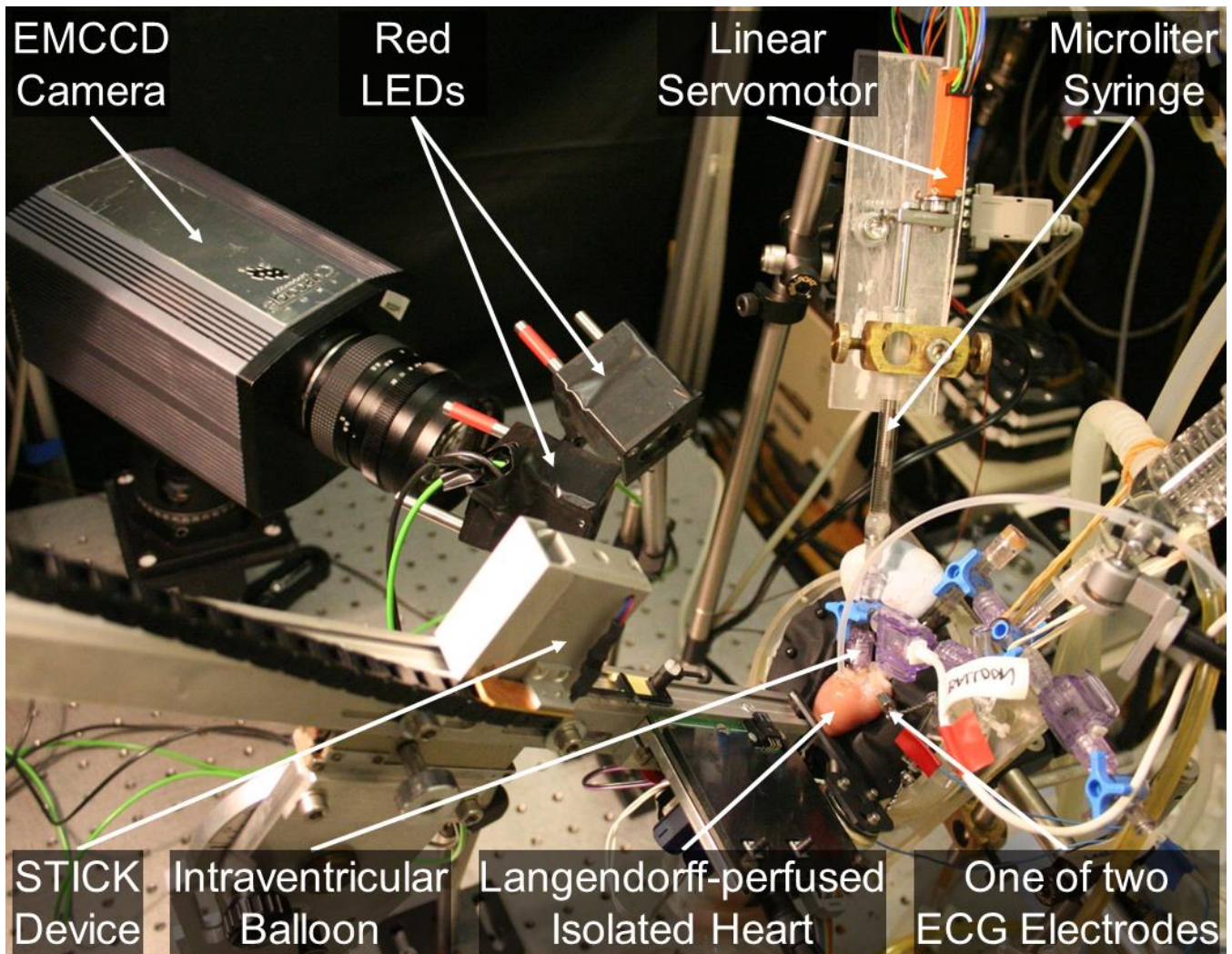


Figure S2. Effect of ATP-inactivated potassium (K_{ATP}) channel blocker glibenclamide on left ventricular repolarization. Representative optical mapping data for 90% repolarization time before (A) and after (B) application of 10 μ M glibenclamide (0 ms represents earliest repolarization in the associated map). Isochrones represent 2 ms steps.

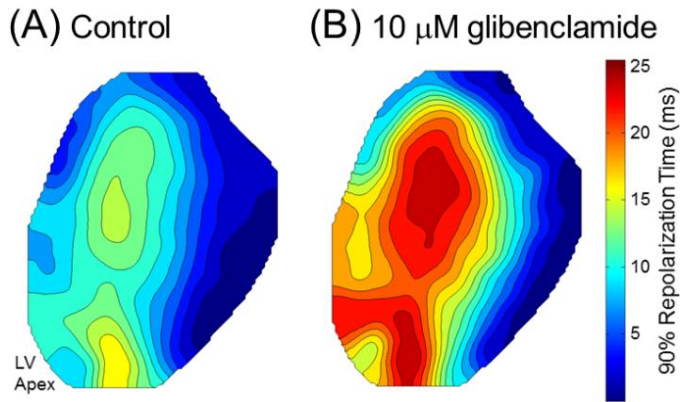
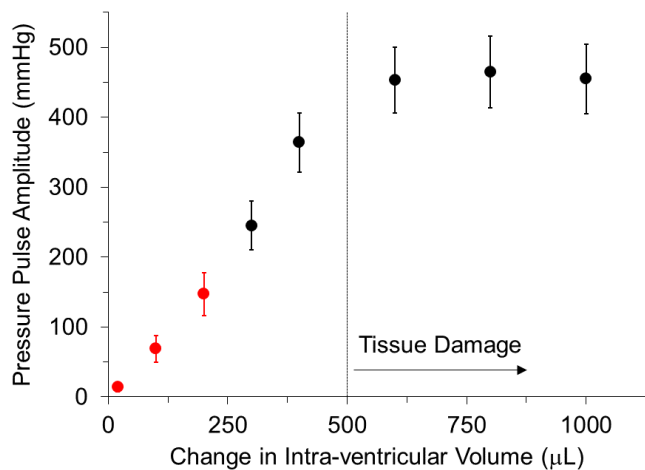
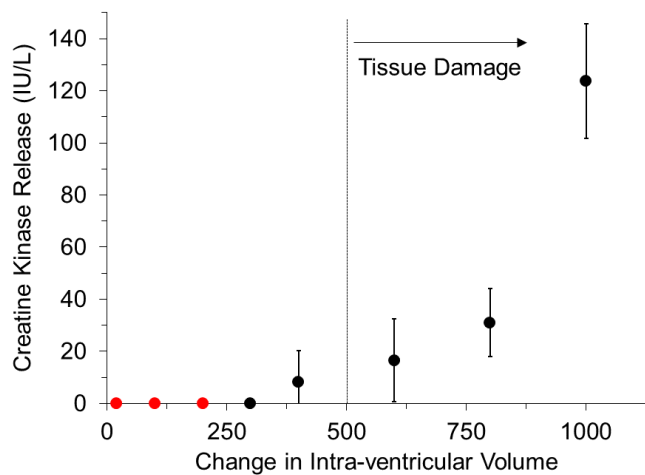


Figure S3. Intra-ventricular volume pulse-induced tissue damage, assessed by creatine kinase release. Relationship between intra-ventricular balloon volume pulse and peak-pressure (**A**) or creatine kinase release (**B**, averaged from $n = 6$ hearts). Standard experiments employed 20 - 200 μL volume pulses (red); gray vertical line indicates volumes beyond which tissue damage is unavoidable.

(A) Pulse amplitude-volume relationship



(B) Tissue damage-pulse volume dependence



SUPPLEMENTAL MOVIES

Movie S1. High-resolution, slow motion, bright-field video of sub-contusional, local, epicardial mechanical stimulation. Selected panels presented in Fig. 1A. Videos acquired at 2,000 fps, with playback slowed by 40× (to 50 fps).

Movie S2. Voltage optical mapping video of typical sinus node-induced left ventricular excitation. Selected panels presented in Fig. 2A. Videos acquired at 511 fps, with playback slowed by ~50× (to 10 fps).

Movie S3. Voltage optical mapping video of representative mechanically-induced left ventricular excitation on same heart as in Movie S2. Selected panels presented in Fig. 2B. Videos acquired at 511 fps, with playback slowed by ~50× (to 10 fps). Note that bright region at the mechanical stimulation location, which turns dark during excitation, is an artifact caused by the contact probe entering the field of view.

Movie S4. Voltage optical mapping video of local electrically-induced left ventricular excitation on same heart as in Movies S2 and S3. Selected panels presented in Fig. 2C. Videos acquired at 511 fps, with playback slowed by ~50× (to 10 fps).

Movie S5. Voltage optical mapping video of mechanically-induced left ventricular fibrillation. First segment shows the sinus beat preceding the local mechanical stimulation, the second segment shows sinus excitation and part-repolarization, followed by mechanical stimulation and ventricular fibrillation. Top signal on the right is the surface ECG, bottom signal is the normalized fluorescence (representing transmembrane potential) averaged from the 2×2 pixel area outlined in blue. Motion artifact with mechanical stimulation accounts for the temporary loss of signal integrity. Same heart as presented in Fig. 3. Note that signal-void in tissue near the apex during contact probe approach and during fibrillation is caused by the shadow of the retracted probe. Videos acquired at 511 fps, with playback slowed by ~50× (to 10 fps).