Cardiac Tissue Slices: Preparation, Handling, and Successful Optical Mapping

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Abstract

Cardiac tissue slices are becoming increasingly popular as a model system for cardiac electrophysiology and pharmacology research and development. Here, we describe in detail preparation, handling and optical mapping of trans-membrane potential and intracellular free calcium concentration transients (CaT) in ventricular tissue slices from guinea pigs and rabbits. Slices cut in the epicardium-tangential plane contained well-aligned in-slice myocardial cell strands (‘fibres’) in sub-epicardial and mid-myocardial sections. Cut with a high-precision slow-advancing microtome at a thickness of 350 to 400 μm, tissue slices preserved essential AP properties of the pre-cutting Langendorff perfused heart. We identified the need for a post-cutting recovery period of 36 min (guinea pig) and 63 min (rabbit) to reach 97.5% of final steady-state values for action potential (AP) duration (APD) (identified by exponential fitting). There was no significant difference between the post-cutting recovery dynamics in slices obtained using 2,3-butanedione 2-monoxime or blebbistatin as electro-mechanical uncouplers during the cutting process. The rapid increase in APD after cutting was mainly caused by the exposure to ice-cold solution during the slicing procedure, not by tissue injury, differences in uncouplers, or pH-buffers (bicarbonate; HEPES) used. To characterise intrinsic patterns of CaT, AP, and conduction, a combination of multi-point and field stimulation should be used to avoid misinterpretations based on source-sink effects.

In summary: we describe in detail the preparation, mapping, and data analysis approaches for reproducible cardiac tissue slice-based investigations into AP and CaT dynamics.

Keywords (4-6 words)

Heart; Optical mapping; Multi-parametric; High spatial resolution; Voltage and calcium sensitive dyes, Live tissue slices

Introduction

Live tissue slices are well-established pseudo-two-dimensional (2D) models for research into organ (patho-)physiology and drug effects, in particular for brain (54) and liver (29). In cardiac research, tissue slices have received less attention, compared to models such as the Langendorff-perfused whole heart, arterially-perfused tissue wedges, cell cultures, or isolated single cells (21). Nonetheless, cardiac tissue slices represent a unique model for cardiac electrophysiology (20), also opening up new possibilities for pharmacological research and development (19, 21). Cardiac tissue slices permit the observation of different functional parameters such as trans-membrane potential ($V_m$) and calcium transients (CaT) and their correlation to cellular substrates, which is often more difficult in three-dimensional (3D) model systems. Compared to single isolated cells or cell culture, slices benefit from inclusion of the various cell types that make up native tissue such as myocytes and fibroblasts in myocardium (12). The locally preserved cell-cell electrical and mechanical connections (39, 72) and the extracellular matrix reflect a more in vivo like profile than other 2D or lower dimensionality models (13).
The use of cardiac tissue slices in metabolism research dates back at least eight decades to the work by Pincus (58) and Thienes et al. (70, 71). This technology has been applied to the measurement of oxygen consumption (66, 74), assessment of ATP-sensitive potassium channel (K\text{ATP}) contributions to ischaemic preconditioning (34), electrophysiological studies of re-entry induction by premature stimuli (56), and drug testing (9, 10).

Numerous technical difficulties obtaining consistent vital cardiac slices with uniform thickness and minimal tissue damage, including slow vibratome blade advance speeds (50 μm/s or less), minimal z-axis blade deflection (<1 μm), slice thicknesses, and alignment of the cutting plane. In previous reports, cardiac slice thicknesses varied from 150 μm (33) to 500 μm (20, 56). In order to maintain sufficient oxygen diffusion to all cells inside the section, investigators now favour slices of no more than 400 μm thickness (3), to avoid potential exposure to hypoxic conditions. As suggested by Yashura et al. in the 1990s (74), cutting ventricular tissue tangentially to the epicardial surface allowed for an optimised alignment of (at least near-epicardial) slices with locally prevailing cell orientation, compared to transmurally-directed sections. This was confirmed by Bussek et al., comparing the results of different cutting directions using 2-photon microscopy and histology (10).

Previous studies used different recovery protocols, with durations ranging from 30 min (7) to two hours (74), and with different buffer solutions. No detailed information about the influence of post-cutting recovery protocols on electrophysiological measurements has been published to date.

Most published electrophysiology studies on cardiac tissue slices have obtained relatively low spatial resolution data, from point-recordings by patch clamp (7) or sharp electrodes (6, 8, 33), to 60 channel multi-electrode arrays (MEA; (10, 11)). Few studies (20, 45, 56) have used high spatial resolution optical methods to monitor electrophysiological parameters. This includes our proof-of-principle-studies (69) and (45), in which we employed dual V\text{m} and CaT mapping of ventricular slices to illustrate the suitability of the approach for studying the effects of mechano-electric feedback (MEF) and for dynamic monitoring of drug effects. Different camera types have been used for optical mapping of V\text{m} and CaT, most commonly CMOS (28, 41, 63) and EMCCD cameras (43). In cardiac tissue slices, the signal is strong enough to be successfully recorded by cost-effective EMCCD cameras. For more detailed reviews of cardiac optical mapping approaches, please refer to articles by Herron et al. (35) and by Entcheva and Bien (23). Cameras with more than 10,000 pixels and with a frame rate in the kHz-range acquire enormous amounts of data. Processing and analysing of these data can be challenging. Freely available programs like ImageJ (27, 61) are helpful, and more sophisticated methods have been developed for processing optical imaging data obtained from single cells and whole heart (42, 67, 75).

The aim of this study was to refine experimental conditions for successful tissue slice preparation, optical mapping, and data analysis. Attention is drawn to recovery times, temperature effects, and buffer solution composition. We describe an approach for semi-automated data analysis, which achieves reliable extraction of information on AP and CaT parameters. The utility of these recommendations is illustrated using recordings from rabbit and guinea pig cardiac tissue slices.

### Materials & Methods

#### Heart Isolation

Male New Zealand White rabbits (1-2 kg, N = 9) and female guinea pigs (250-400 g, N = 3) were humanely killed, either by anaesthetic overdose (pentobarbital, 70 mg/kg for rabbits) or cervical dislocation (guinea pigs), in accordance with Schedule 1 of the UK Home Office Animals (Scientific Procedures) Act 1986. Hearts were quickly excised and perfused in Langendorff-mode with bicarbonate-buffered solution (containing, in mmol/L: NaCl 123, CaCl\text{2} 1.8, KCl 5.4, MgCl\text{2} 1.2, NaH\text{2}PO\text{4} 1.4, NaHCO\text{3} 24, Glucose 10; bubbled with 95% O\text{2} / 5% O\text{2}; pH 7.4 at 35±2°C). For electro-mechanical uncoupling, blebbistatin (10 μmol/L, Ascent Scientific, Cambridge, UK) was
added after dye loading and before slicing / optical mapping. All chemicals were obtained from Sigma-Aldrich (Dorset, UK), unless otherwise stated. Note that fat accumulation at the epicardial surface increases with animal age. Fat tissue is difficult to cut and can blunt the blade, so we recommend removal of fat tissue before slicing.

### Dye Loading

#### Rabbit

Fluorescent dyes were loaded via the coronary circulation, applied by injection into the aortic cannula. First, 22 µL of a solution containing the voltage-sensitive dye di-4-ANBDQPO (20 µL of stock solution 27 mmol/L in ethanol, University of Connecticut Health Center, USA) and Pluronic F-127 (2 µL of a 20% stock solution in dimethyl sulfoxide (DMSO); Life Technologies, Paisley, UK) was slowly added over a 4–5 min period (i.e. at a Langendorff perfusion rate of 16-20 mL/min, the dye was diluted in ~65-100 mL bicarbonate-buffered solution during application). To improve calcium dye loading and retention of the dye in the cytoplasmic matrix, rabbit hearts were pre-perfused with bicarbonate-buffered solution containing 0.5 mmol/L probenecid to prevent dye-leakage from the cytoplasmic space into the extracellular medium (22). The Ca²⁺-sensitive dye Rhod-2-AM (200-250 µL stock solution, 1 mg/mL in DMSO; AAT Bioquest Inc., Sunnyvale, USA) was added over a 5 min period, and the dye-containing solution (~70-100 mL) was re-circulated for 40 min. After completion of dye loading, hearts were perfused with bicarbonate-buffered solution to wash out any excess voltage- and Ca²⁺-sensitive dyes.

#### Guinea pig

Langendorff-perfused guinea pig hearts were loaded with 22 µL of a solution containing the voltage-sensitive dye di-4-ANBDQBS (20 µL 29 mmol/L in ethanol, University of Connecticut Health Center, USA) and Pluronic F-127 (2 µL of a 20% stock solution in DMSO; Life Technologies, Paisley, UK) through bolus injection over 4–5 min (at a Langendorff perfusion rate of 8-10 mL/min, the dye was diluted in 40-50 mL bicarbonate-buffered solution during application). The Ca²⁺-sensitive dye Cal-520-AM (200 µL, 1 mg/mL in DMSO; AAT Bioquest Inc.) was loaded after the voltage dye via bolus-injection, and recirculated for 40 min as described above. Cal-520-AM has a spectrum very similar to Fluo-4, with an improved signal-to-noise ratio (48).

### Tissue Slice Preparation

It is essential to keep tissue immobilised during vibratome cutting. This reduces tissue damage, caused by movement relative to the cutting plane. In previous studies, 2,3-Butanedione 2-monoxime (BDM) (31) was applied as electro-mechanical uncoupler (9-11), while blebbistatin is currently more widely-accepted (65) for optical mapping (25). Both uncouplers were tested. After dye-loading, hearts were perfused at room temperature with either BDM-containing HEPES-buffered solution (in mmol/L: NaCl 140, CaCl₂ 1.8, KCl 5.4, MgCl₂ 1, Glucose 11, HEPES 5, BDM 10, and probenecid 0.5 for Rhod-2-AM loaded hearts; bubbled with 99.9% medical grade O₂; pH 7.4) or blebbistatin-containing (10 µmol/L) bicarbonate-buffered solution (contents described as above, with 0.5 mmol/L probenecid for Rhod-2-AM loaded hearts), until the heart did not show any contractions.

The left ventricular free wall was removed from the heart, the apex was cut off at about 1/8 of the total length of the heart. A cut was made counter clockwise from the apical end along the LV-septum border. This cut was continued below the circumflex artery along the coronary sulcus, and then turned towards the apex after covering about 2/3 of the left ventricular free wall width. Edges and any papillary muscles present were trimmed to enable flattening of the excised tissue, before gluing the endocardium-down (histoacryl tissue adhesive; Braun, Melsungen, Germany) onto a block of 4% agar (low melting-temperature agar, Nusieve GTG agarose, Lonza, NL), which in turn had been fixed on top of the vibratome cutting stage. The tissue block was cut in the epicardium-tangential plane, using a high precision vibratome (7000smz tissue slicer, Campden Instruments Ltd., Loughborough, UK).
with a ceramic blade (Campden Instruments Ltd.) at a progression speed of 0.03 mm/s (lateral blade vibration amplitude 2 mm, frequency 80 Hz). Slices were cut at a thickness of 350 to 400 μm. This was chosen to avoid hypoxic conditions in the tissue core, while preserving a good source of fluorescent signals. During the slicing procedure, the tissue was kept in ice-cold oxygenated BDM-containing HEPES-buffered solution (bubbled with 99.9% medical grade O₂; pH 7.4 at 4°C) or blebbistatin-containing (10 μmol/L) bicarbonate-buffered solution (bubbled with 95% O₂/5% CO₂; pH titrated to 7.4 at 4°C).

Tissue slices were collected on thin blocks of Polydimethylsiloxane (Sylgard 184, Dow-Corning, Midland, MI USA), and held in position using a plastic-framed soft mesh. These assemblies were incubated in blebbistatin-containing bicarbonate-buffered solution for tissue recovery at 35±2°C. For the assessment of the minimum recovery time needed to reach steady-state AP properties, measurements were taken at several time points after sectioning (from 5 min up to 3 h).

In a separate set of experiments, designed to explore the influence on AP properties of incubation temperature, rabbit cardiac tissue slices which had reached AP steady-state after the initial incubation in warm (35±2°C) bicarbonate-buffered solution were re-exposed to ice-cold BDM-containing HEPES buffer for ~1 h before moving them back to warm (35±2°C) blebbistatin-containing bicarbonate-buffered solution for repeat-measurements. $V_m$ and $\text{CaT}$ signals were recorded at multiple time points before and after re-exposure to cold.

**Dual $V_m$ and $\text{CaT}$ Mapping of Tissue Slices**

For imaging, tissue slices were kept in blebbistatin-containing bicarbonate-buffered solution at 35±2°C. A LED (LED-CBT-90-R, peak wavelength 624 nm; Luminus Devices, Billerica, USA) with excitation-filter D640/20x was used for excitation of the $V_m$ sensitive dye. A white light illuminating LED (LED-CBT-90-W; Luminus Devices) was used for excitation of the $\text{Ca}^{2+}$-sensitive dye, using excitation-filter S555/25x for Rhod-2-AM (rabbit) or D470/40x for Cal-520-AM (guinea pig). All filters were obtained from Chroma Technology (Bellows Falls, USA) and LED light collimated with a plano-convex lens (LA1951; Thorlabs, Ely, UK).

The $V_m$ and $\text{CaT}$ signals were collected by an EMCCD camera (Cascade 128+; Photometrics, Tucson, USA). For rabbit tissue slice mapping (di-4-ANBDQPQ & Rhod-2-AM), a custom-made multi-band filter ET585/50-800/200 (Chroma Technology) and a long-pass filter with significant transmission at wavelengths >575 nm (BLP01-561R-25; Semrock, Rochester, USA; were placed in front the camera lens (Navitar, Rochester, USA) for collection of fluorescence emission. For guinea pig slices (di-4-ANBDQBS & Cal-520-AM), a different custom-made multi-band filter was used (ET525/50-800/200; Chroma Technology). The dual $V_m$ and $\text{CaT}$ imaging setup relied on the excitation isosbestic-points for the voltage responses of di-4-ANBDQBS and di-4-ANBDQPQ, respectively (44, 46).

The EMCCD camera was used at its maximum resolution (128x128 pixels) and at a frame rate of 510 Hz. $V_m$ and $\text{CaT}$ data was obtained pseudo-simultaneously using one camera, $V_m$ or $\text{Ca}^{2+}$ dye excitation LED were switched on in a non-overlapping frame-accurate 1:1 sequence (43). Each parameter was sampled with a frequency of 255 Hz. Linear interpolation was used to estimate data values between two consecutive measurements for each parameter to enable comparison of temporal correlation.

**Pacing**

Four point-electrodes (Lohmann Research, Castrop-Rauxel, Germany) and one set of parallel platinum electrodes were coupled to a tailor-made electrical stimulator for local and field stimulation, respectively. The four point-electrodes were gently placed onto different locations of the tissue slices, as far from one another as possible. The two platinum electrodes (20 mm x 2 mm x 0.2 mm, placed approximately 3.5 cm apart from each other) were aligned in parallel to the apex-base direction of the slice. We recorded $V_m$ and $\text{CaT}$ signals at 1, 2, 3, 4 and 5 Hz pacing; 15 APs for each frequency were recorded for each point stimulation site and for field stimulation. The stimulus amplitude was chosen
to be 1.5 times the threshold voltage; pulse duration was 2 ms, using a bipolar stimulus to avoid
deleterious electrochemical effects.

**Whole Heart Epicardial Imaging**

Optical mapping on hearts (rabbit: n = 2; guinea pig n = 2), was performed prior to and in-between
(below) slice collection, to assess the dye loading and to collect functional parameters from sub-
epicardial regions in the Langendorff perfused intact heart. During whole heart imaging, the heart was
perfused with blebbistatin-containing bicarbonate-buffered solution, with the left ventricular free-wall
facing the camera. Light source, excitation, and emission filters were the same as described above for
tissue slice imaging in the two species, respectively.

**Collecting Sub-Epicardial Slices from Langendorff perfused Heart**

Guinea pig hearts were isolated, dye-loaded, perfused with bicarbonate buffer solution, and were
placed into the modified slicing chamber of a vibratome (Campden7000smz). The chamber was filled
with solution (35±2°C) and the epicardial $V_m$ was optically mapped. Cardiac motion was restricted by
perfusion with 10 μmol/L blebbistatin, while the heart was fixed gently in a silicone cradle. A cannula
(Gauge 16) was placed through the left ventricular wall to avoid intra-ventricular fluid build-up.
Tissue slices (~400 μm) were cut tangentially to the mapped epicardial surface. The exposed surface
was optically re-mapped, before each new cut. This process was repeated until the left ventricular wall
was completely sectioned. Slices were mounted as described earlier and transferred to an imaging
chamber (35±2°C) for optical mapping. Field stimulation was used for whole heart and tissue slice
imaging.

**Assessment of Viability and Structure of Cardiac Slices Electron Microscopy**

To assess the ultrastructural integrity of cardiac tissue slices, both transmission electron microscopy
(TEM) and scanning electron microscopy (SEM) were used. Tissue was fixed with 2.5% buffered
 glutaraldehyde (Agar Scientific, Stansted, United Kingdom), post-fixed with OsO₄, dehydrated in
 acetone, and embedded for TEM in Epon. Samples for SEM were prepared in a similar manner,
 except they were critically point-dried and sputter-coated with gold. TEM samples were thin-
 sectioned in the plane of the slice and examined on a JEOL 1200EX (Welwyn Garden City, United
 Kingdom). SEM samples were examined on a JEOL 35 SEM (Peabody, USA) at 15kV.

**Data Analysis**

Due to the large amount of data collected in each experiment, a semi-automatic data analysis tool was
written in Matlab® (The MathWorks, Natick, USA), which analyses data sets to extract relevant
information. The details for $V_m$ signal processing and parameter estimation are shown in Figure 1 as a
flowchart; CaT signal processing was conducted in a similar manner.

Signal processing was necessary to reduce the impact of noise upon parameter estimation (e.g. APD
was calculated from processed $V_m$ signal traces). Recorded signal traces were processed through the
following steps: 1) identifying the depolarisation timing on the filtered trace to break up the recorded
signal trace into individual AP cycles (Figure 1A-C); 2) estimating the maximum ($F_{max}$) and baseline
($F_0$) fluorescence intensities for each identified AP, and calculating the corresponding fractional
fluorescence ($\frac{(F_{max} - F_0)}{F_0} \times 100$, to give the percentage of the fluorescence ratio; Figure
1D); 3) correcting the initially identified depolarisation time point if needed (Figure 1E); 4)
polynomial fitting (1st order for depolarisation phase, 4th order for repolarisation phase) through raw
data points (Figure 1F); 5) linear correction of baseline drift (Figure 1G). Very weak signals (i.e.
where the fractional fluorescence was <0.3%) were classified as ‘no signal’. In the online supplement
an example is shown of raw and processed $V_m$ data from a representative tissue slice (from rabbit; see
Particular care needs to be taken when defining the maximum AP amplitudes, as this feeds through to the calculation of APD, customarily defined at set %-repolarisation levels (relative to maximal depolarisation). Maximum AP amplitude is defined by a single data point, which makes peak values extremely sensitive to noise and sampling rate in experimental measurements. This can give rise to artificially exaggerated differences in AP amplitudes, with consequent spurious changes in APD estimation, even when underlying AP shapes are very similar. To obtain more physiologically-relevant and robust APD data, both for experimental measurements and simulated traces, a more reproducible identification of the AP ‘peak value’ is needed. Here, for each AP signal, we use the 95th percentile of the signal (i.e. the value below which 95% of all data-points of the AP signal fall) as a surrogate for the (apparent) absolute peak value. Figure 1I shows one AP from an optical mapping measurement (pre-processed with procedure described before) with defined maximal value, APD at 50% repolarisation (APD50) and APD at 80% repolarisation (APD80) marked out. For our tissue slice optical mapping data, APD80 was used instead of APD at 90% repolarisation (APD90) to reduce the impact of baseline noise on APD estimation. After calculating APDs for each pixel, APD maps were generated (averaged APD over a number of AP cycles). An example of APD80 and APD50 maps is shown in Figure 1I (note: grey colour coding is used to identify areas within the physical dimensions of a slice, where insufficient fractional fluorescence was observed; classified as ‘no signal’, see above). The threshold for ‘no signal’ classification can be adjusted, according to experimental needs. 

Fluorescence signal strength depends on dye loading, tissue integrity/viability and underlying structures (e.g. cleavage planes between cell layers). It is common to have uneven signal strengths in cardiac tissue, including slices, and the size of ‘no signal’ areas varies. 

To characterize AP shape quantitatively in an automated manner, two indicators were used. The first is the area under AP curve, normalised to the rectangle defined by AP amplitude (95th percentile) and structures (e.g. cleavage planes between cell layers). It is common to have uneven signal strengths in cardiac tissue, including slices, and the size of ‘no signal’ areas varies.

Together, AP area ratio and AP skewness provide user-independent and quantitative characterisations of AP shape (in terms of parameters such as rectangularity or plateau) allowing for reliable and automated identification of changes in AP shape after an intervention, and comparison of AP shapes between individual samples. From the regionally resolved upstroke timing in the processed data, conduction velocity was estimated using Bayly’s method (5), and the activation wave-front speed was calculated from velocity vectors identified based on activation timing across the slice (see Figure 1L).

Before processing each signal trace, spatial filtering (such as mean or Gaussian) can be applied to the optical mapping images. In our case, a mean filter with a range of 3 pixels was applied (i.e. the averaged value of a 3x3 pixel square was taken as the new value of the central pixel in this square). After spatial filtering, the signal trace (i.e. fluorescence intensity change over time) was read out for each pixel (in the case of dual $V_m$ and CaT mapping, $V_m$ and CaT traces were separated) and processed with the methods shown in Figure 1A-H. Functional parameters (e.g. APD and AP shape indicators) were calculated for each processed AP (or CaT) from the signal trace (as shown in Figure 1I-L). These functional parameters were then grouped by pacing frequencies, and for each pacing frequency the average functional parameter value was identified (for example, 15 steady-state APs were recorded at 2 Hz pacing, and the representative APD value was the average of the 15 APDs). Functional parameter maps (like the APD80 maps shown in Figure 1I and the AP skewness map shown in Figure 1K) were plotted for each pacing frequency, with each point on the map representing the averaged functional parameter value per slice.
Statistical Analysis

To quantify the post-cutting recovery time, an exponential curve:

$$APD80_{normalised} = A(1 - e^{-Bt})$$

was fitted through the normalised $APD80$ data (mean $APD80$s for the whole slice), obtained at several post-cutting time points ($APD80$ at each post-cutting time point is normalised to the maximal $APD80$ value observed over the entire time interval). In the formula, $A$ and $B$ are constants to be fitted and $t$ is the time (in min) after removing the slice from ice-cold solution. The fitting was performed using Matlab curve fitting tools.

To compare the recovery dynamics of the two species (i.e. rabbit and guinea pig) and to assess any difference in the recovery dynamics caused by the use of different solutions (i.e. BDM-containing HEPES buffer, blebbistatin-containing bicarbonate buffer), both F-tests and T-tests were performed.

For the F-test (55), the null hypothesis is that one curve can fit through all data points (two species, two solutions) and that this fit is as good as fitting two groups of data points separately. The formula for F ratio calculation can be found in (55).

The T-test is performed to assess whether two groups of data have two significantly different recovery times (time to reach steady-state AP). We concentrate on the assessment of the fitted constant $B$ (without extra assessment of $A$), since the recovery time is dependent only on $B$. For example, the time needed to reach 97.5% of the final AP steady-state equals $\frac{\ln(0.025)}{-B}$. The $t$ value is calculated (in a non-standard manner) as:

$$t = \frac{|\hat{B}_1 - \hat{B}_2|}{\sqrt{SE_1^2 + SE_2^2}}$$

where $\hat{B}_1$ and $\hat{B}_2$ are the best-fit of constant $B$ for groups one and two, respectively, and $SE_1$ and $SE_2$ are the standard-error of the fitted constant $B$ for groups one and two, respectively (this can be read-out from Matlab regression tool). The total number of degrees of freedom is the sum of degrees of freedom of the two groups. After calculating the $t$ value, the P value can be obtained.

Results:

Dual $V_m$ and CaT Measurements

Two dye combinations were used for dual $V_m$ and CaT mapping of tissue slices: di-4-ANBDQPO in combination with Rhod-2-AM for rabbit, and di-4-ANBDQBS with Cal-520-AM for guinea pig heart. Figure 2 shows the raw signals, acquired from one rabbit (subplot A) and one guinea pig (subplot C) left ventricular tissue slice. Figure 2B and D show 2D maps of the progression of $V_m$ (top) and CaT activation waves (bottom) after point-stimulation at locations identified in panel A. The delay in onset of CaT activation, compared to start of AP upstroke, was $2.21 \pm 0.60$ ms in rabbit (mean ± standard deviation (SD); 2 animals, 5 slices) and $2.33 \pm 1.00$ ms in guinea pig (mean ± SD; 2 animals, 9 slices). No significant difference (with 5% significance level) in AP to CaT activation was identified between the two species ($P = 0.81$).

Pacing frequency-dependent responses of APD and CaT duration, as described before (60), were observed in both species (shortening of both APD and CaT at higher pacing rates; data not shown).

Time to Reach Equilibrium After Slicing
In both species, sharp, short, and triangular APs without a clear plateau phase were observed immediately after cutting the tissue in ice-cold solution, both for BDM-containing HEPES buffer and blebbistatin-containing bicarbonate buffer. Figure 3A-1 and 3A-2 (guinea pig) and 3A-3 (rabbit) showed unfiltered (unprocessed), normalised V_m signals (averaged over the whole slice), recorded immediately after cutting, and subsequent to incubation at body temperature for over 1 h. Figure 3B-1, 3B-2 and 3B-3 show APD80 histograms (2 Hz pacing) of the same guinea pig and rabbit slices as shown in 3A-1/2/3, measured at several post-cutting time points. An increase in APD80 was observed after cutting, until a steady-state was reached. In Table 1, AP descriptors (AP area ratio and skewness) of a guinea pig slice (AP signal in Figure 3A-1) measured at two post cutting time points (2 and 66 min), together with the AP descriptors obtained from the same heart (LV epicardial view) prior to cutting. Compared to the AP obtained shortly after cutting, APs recorded after 1 h incubation had a shape much closer to that measured in the whole heart before sectioning.

**Table 1:** Example of AP shape indicators, obtained from a guinea pig tissue slice before and after cutting. N numbers used to calculate mean and SD values are the number of pixels capturing V_m signal from the guinea pig tissue slice or the guinea pig whole heart epicardial surface. N number equals to 3,547 for the slice and 8,428 for the whole heart.

<table>
<thead>
<tr>
<th></th>
<th>Average AP area ratio</th>
<th>Average AP skewness</th>
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<tbody>
<tr>
<td>Guinea pig whole heart:</td>
<td>0.751 ± 0.08</td>
<td>-1.157 ± 0.061</td>
</tr>
<tr>
<td>before cutting (epicardial aspect of LV)</td>
<td></td>
<td></td>
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<tr>
<td>Guinea pig tissue slice:</td>
<td>0.567 ± 0.076</td>
<td>0.010 ± 0.243</td>
</tr>
<tr>
<td>2 min after cutting</td>
<td></td>
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<tr>
<td>Guinea pig tissue slice:</td>
<td>0.718 ± 0.012</td>
<td>-0.960 ± 0.075</td>
</tr>
<tr>
<td>66 min after cutting</td>
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</table>

Quantification of the post-cutting recovery time needed to reach steady-state AP properties, an exponential curve was fitted through the normalised APD80 values, measured at different post-cutting time-points. The time to reach 97.5% of the final- steady-state APD80 values was calculated.

Whether transient exposure to BDM and/or HEPES buffer (guinea pig, 3 slices, 2 animals) during slice preparation was a major contributor to the AP shortening and triangular shape seen after cutting, the process was repeated in ice-cold bicarbonate-buffered solution containing blebbistatin (guinea pig, 5 slices, 2 animals). Note that all slices, whether cut in BDM or blebbistatin-containing solution, were re-warmed in the same buffer (blebbistatin-containing bicarbonate buffer immediately after cutting), to focus on cutting conditions. Neither F- nor T-test identified any differences in AP recovery dynamics or time after the slicing procedure (P = 0.725 for F-test; P = 0.096 for T-test).

Changes in normalised APD80 (mean APD80 over the whole slice), measured at several post-cutting time-points from guinea pig (8 slices, 4 animals) and rabbit (7 slices, 4 animals) slices, respectively, during 2 Hz field stimulation (Figures 4 A and B) The fitted exponential curves are shown in red. The time to reach 97.5% of steady-state APD80 values was 36 min and 63 min for guinea pig and rabbit, respectively. The F-test identified a significant difference between recovery dynamics of the two species at 5% significance level (P = 0.006) and the T-test identified a significant difference between the recovery times of the two species at 5% significance level (P = 0.008).

Changes in AP shape are most likely due to gradual recovery following sectioning. We saw no evidence to suggest that BDM or HEPES affect AP shape. However, in slices obtained by sectioning guinea pig Langendorff perfused heart at 37 °C, we did not observe sharp and short APs shortly after cutting (Figure 5). AP shapes closely resembled those collected from the tissue prior to cutting. Figure 5A shows the APD80 maps from the left ventricular epicardium/sub-epicardium of a Langendorff perfused heart and from the subsequently taken sub-epicardial tissue slice, here at 4 Hz field stimulation. In Figure 5A the red outline on top of the whole heart APD80 map indicated the area where the next slice was taken. The APD80 histograms of the area inside the red outline in the
perfused whole heart and from the subsequently taken tissue slice, here mapped 8 min after cutting, are shown in Figure 5B. In Figure 5C, averaged AP traces are shown from the sub-epicardial region inside the red outline of the perfused heart before cutting (black) and of the subsequently taken tissue slice (red). The slices and the whole-heart were paced by field stimulation. In the whole heart, the field simulation was not sufficient to activate the whole heart at the same time; there was still a gradient of activation time through the ventricular wall. Although a blue light source was used to reduce light penetration into the tissue, signal from deeper myocardial layers were also collected, which explain why the AP upstroke appears slower in the whole heart than in the pseudo-2D slice.

In Figure 6 we show spatially averaged AP traces (2 Hz field stimulation) of a rabbit slice at steady-state after the initial sectioning (79 min post-cutting), and at 5 min and 18 min after re-exposure to ice-cold solution (for 60 min); all recordings at body temperature. Shortly after starting the re-warming process (5 min), a short and sharp AP is observed, which shows a trend towards increasing APD over time (18 min). This illustrates that exposure to low temperature appears to play a critical role in causing the observed changes in AP shape and duration.

**Multiple Point Stimulation**

Previous cardiac tissue slice studies tended to use one site for AP stimulation (10, 11, 57). To explore whether pacing location affects functional parameters (e.g. APD80), we used four different point stimulation sites on each of the slices, and field stimulation as a reference case. APD80 maps obtained with these protocols were compared. To exclude effects from the initial changes associated with tissue recovery (above), data for these comparisons were obtained after at least 70 min storage at body temperature. Measurements were repeated over the course of an hour, with each set of observations consisting of 5 measurements (4 point and 1 field stimulation) taken in swift succession.

The pacing-site dependent differences in APD were observed in most slices, at all the pacing frequencies tested. Differences could be pronounced, as shown in Figure 7, where pacing from one end of the slice yielded a region of very short APD that was absent upon pacing from the other end (compare Figure 7A-1 and 7A-2). Conduction velocity (Figure 7B-1; vectors plotted on top of the depolarisation time map, where the size of the vector is proportional to the magnitude of the conduction velocity) and conduction speed maps for the first pacing location (Figure 7C-1) show that slowed conduction coexists with the short APD. When pacing from the other end, both conduction velocity and speed (Figure 7B-2 and 7C-2) were more inhomogeneous, matching the APD distribution.

**Assessment of Tissue Slice Viability**

To assess ultra-structural integrity of tissue slices, SEM and TEM were conducted on samples from tissue slices at ~20 μm below the sectioned surface. The TEM in Figure 8 illustrates that sub-cellular structures, including well-aligned contractile fibres, intact mitochondria and nuclei during a 4 h period after cutting, were normal in appearance. Spatial relationships of myocytes, extracellular collagen, fibroblasts and vasculature all appeared normal by SEM.

**Discussion**

**Dual V_m and CaT Optical Mapping**

The main advantages of optical mapping are high spatial resolution, capacity to monitor multiple physiological parameters, and the ability to do so simultaneously. In addition, voltage-sensitive dyes can record AP shapes rather than activation and repolarisation timings only.

Dual V_m and CaT mapping has been used frequently for whole heart studies (15, 17, 18, 50), but has had limited application to cardiac tissue slices (45). V_m and CaT are key parameters of cardiac function and their relationship is crucial for electro-mechanical coupling and mechano-electric feedback in the heart (52). Combined V_m and CaT optical mapping allows one to study not only the individual characteristics of these two parameters (e.g. the frequency response of V_m and CaT), but
also their spatio-temporal interrelation (16). Monitoring both parameters simultaneously can be beneficial for testing pharmacological interventions, which may affect \( V_m \) and \( C_{aT} \) independently. Finally, dynamic high-resolution observation allows one to identify conduction wave-front maps and spatial distribution of key parameters (e.g. APD), which is crucial, for example for studying ventricular fibrillation (4, 14).

We used two dye-combinations for combined \( V_m \) and \( C_{aT} \) mapping, and demonstrated their utility in two species (Figure 2). These combinations were not species-specific, but application to other species could require modification of excitation and emission filters since the excitation isosbestic-points of di-4-ANBDQPO and di-4-ANBDQBS vary (e.g. the excitation isosbestic-point of rabbit is at a longer wavelength compared to rat) (46).

Both di-4-ANBDQPO and di-4-ANBDQBS are voltage-sensitive dyes of an increasingly wide range of dyes with different spectral characteristics (73), that combine significantly improved \( V_m \) signal with internalisation stability (53). These characteristics make them ideal for tissue slice optical mapping, as the thin tissue preparations yield less signal compared to arterially-perfused large tissue blocks.

Both Rhod-2-AM and Cal-520-AM have relatively high calcium affinities (\( K_d \) ~570 nM and ~320 nM, respectively; data sheets from AAT Bioquest). High-affinity dyes bind calcium during early systole. Slow release of calcium from these dyes may generate inaccuracies in calcium amplitude and apparent calcium decay dynamics (40). Figure 2 shows that both \( C_{aT} \) traces display a fast and slow decay phase; the slow decay phase may, in part at least, be a consequence of relatively slow calcium unbinding from the dye. Further investigations of \( C_{aT} \) properties, including comparisons of \( C_{aT} \) signals recorded with high- and low-affinity calcium dyes, would be necessary (40).

Cardiac Tissue Slices

Although cardiac slices are not as popular yet as isolated cell or Langendorff heart preparations, their moderate complexity can offer unique insights into cardiac electrophysiology. Considering the complexity of the system and its ability to capture \textit{in vivo}-like phenomena, tissue slices can bridge the gap between those two models. Compared to single cells, slices offer preserved cell-cell connections that enable one to study tissue-level phenomena (e.g. conduction), while the locally preserved extracellular microenvironment avoids potential pitfalls of changes in functional properties due to the loss of extracellular matrix or damage by the cell isolation process. Compared to whole heart, tissue slice is a simpler model, which makes the interpretation of structure-function relationships (e.g. the correlation between cell direction and conduction speed) easier. Tissue slices also allow full access to mid-myocardial tissue, which is difficult in the perfused whole heart. They can be cultured (32) and used over several days, which opens up the possibility of maintaining biopsy material, for example from patients, for long-term investigation (6, 11).

Post-Slicing Recovery Time

There is a critical recovery time-period, required for cardiac slices to reach an electrophysiological steady-state. During our recovery protocol, we observed, both in rabbit and guinea pig slices, short and triangular APs right after cutting (e.g. Figure 3). In addition to the short and sharp triangular APs, tissue slices showed relatively weak fractional fluorescence immediately after removal from the ice-cold solution used during cutting, followed by a rapid increase in signal strength. This occurred largely during the first half hour of the recovery period. To quantify the minimum recovery time in our setting, an exponential function was fitted through the normalised data from guinea pig and rabbit. The recovery times need to reach 97.5% of steady-state APD80 values were 36 and 63 min for guinea pig and rabbit, respectively. These findings are consistent with previous reports (7), which highlighted that the success-rate for electrophysiological measurements on cardiac tissue slices from neonatal rat, using patch clamp, increased after 30 to 60 min of incubation, supporting the frequently chosen slice recovery time of ~1 h (20, 56). F- and T-tests suggest significant differences in the post-cutting recovery dynamics and the recovery time between the two species. This highlights the need to verify the post-cutting recovery time when applying this methodology to other species.
There are several possible explanations for the need to allow a recovery period prior to use of slices:
1) BDM, used by many teams during cutting, may need to be washed-out; 2) some or all of the tissue may have become hypoxic during slicing; 3) tissue slicing is associated with severe damage of cells near the cut surfaces, from which the tissue may need to recover; 4) during slicing, the tissue is kept in ice-cold solution, and the return to body temperature may affect its electrophysiological properties.

To assess the first possibility, we performed slicing in cold bicarbonate-buffered blebbistatin-containing (instead of HEPES-buffered BDM-containing) solution. Similar short and sharp AP configurations, and weak fluorescence signals, were observed immediately after cutting. The recovery dynamics of slices cut in these two different buffers did not show any significant difference. This suggests that the kind of uncoupler and the buffer system are not an issue per se.

The second scenario, hypoxic damage, was countered in as far as that is possible by keeping the tissue in ice-cold oxygenated buffer solution during sectioning. This, together with electro-mechanical uncoupling, reduces metabolic demand. Interestingly, slices, taken from the well-oxygenated outer surface of the tissue block, and those taken later and from deeper transmural planes showed no systematic difference in signal quality and recovery dynamics. Therefore, we conclude that it is unlikely that the observed sharp and short AP configurations arise in direct consequence of potentially hypoxic conditions in the tissue block.

To explore the third and the fourth possibilities (i.e. the tissue damage and the temperature effects), we sliced guinea pig whole hearts during Langendorff perfusion with warm (35 ± 2°C) blebbistatin-containing bicarbonate-buffered solution, to obtain near-epicardial tissue slices. In this setting, no sharp and short APs were observed after slicing. Instead, APs, recorded from these slices shortly after sectioning, showed repolarisation morphology and APD that was very similar to the pre-sectioning intact tissue (Figure 5). These results exclude tissue damage as the primary cause of the post-cutting recovery phenomenon, and suggest that the change in temperature is a probable main contributor.

Effects of temperature on AP properties have been observed before (24), generally reporting AP shortening when lowering the temperature from body to room temperature (36, 51). Less is known about effects on the AP of transient exposure of cardiac tissue to ice-cold solution. In order to further explore whether the ice-cold temperature the tissue experiences during slicing is the main reason for the sharp and short APs, observed shortly after cutting, fully-recovered slices with normal AP configurations (n = 3) were re-exposed to ice-cold BDM-containing HEPES-buffered solution for a period of 60 min, and then mapped again. Indeed, the sharp and short AP shape, seen during the initial recovery phase, was observed again (Figure 6). We conclude, therefore, that the use of ice-cold solution, necessary to avoid ischaemic conditions in the tissue block during slice preparation, is a key contributor to the transiently abridged AP configurations, and the observed electrophysiological recovery dynamics.

One possible explanation for these effects could be changes in cell excitability at low temperature. Only part of the cells in a slice may be excited shortly after removal from ice-cold solution, with the portion of excitable cells increasing over time in warm solution. The non-excited cells would then act as current sinks, and therefore truncate the AP. The relatively weak signal shortly after sectioning is in keeping with this possibility, as discussed by others (30).

For slices collected from whole-heart at body temperature, no post-cutting recovery time was required to record near steady-state APs. However, slicing whole-heart at body temperature has its limitations. As sectioning progresses, coronary arteries (which run initially at the epicardial/sub-epicardial surface) get cut, and perfusion of the heart is disturbed. At body temperature, where metabolic demand is relatively high, the heart may quickly enter ischaemia even after cutting just one slice. Therefore, this approach is not suitable for studying multiple transmural slices.

**Frequency Response and Upstroke Time Delay Between AP and CaT**

Shortening of APD with increasing pacing frequency is a well-known response of cardiac muscle (2, 26), and shortening of CaT duration has also been observed (38). Rate-dependent reductions in APD and CaT duration were seen in our LV tissue slices, both from guinea pig and rabbit.
The delay between onset of upstroke of AP and CaT has been measured to be just over 2 ms, both for guinea pig and rabbit (non-significant difference between species). Values in the same order of magnitude have been reported before (47, 49). Longer (17, 62) delays in CaT upstroke (compared to AP upstroke) have also been observed. One reason for this discrepancy is related to the method used to identify the different time points to calculate the CaT delay. These range from ‘earliest onset’ of AP and CaT upstroke (62) to time points corresponding to the AP and CaT peak values (43). Differences may also stem from different measurement techniques (patch clamp (47) vs. optical mapping (49)), use of calcium sensitive dyes with different sensitivity, or use of uncouplers (such as BDM) that affect the L-type calcium channel (1, 31, 37). Overall, the delays reported here are in agreement with values reported using patch clamp (47), optical mapping (49), and computational simulation (68).

Multiple Point Stimulation

AP shape, APD distribution, and conduction dynamics at any given location in the slice can differ, depending on pacing site (Figure 7). A probable explanation for this is the presence of non-uniform source-sink relations in the tissue, which has previously been shown to induce differences in conduction and APD in cell culture (76) and whole heart (64). In slices, there is the additional effect of variable cell alignment relative to the plane of the section. Although an epicardium-tangential cutting plane maximises the proportion of myocytes that are aligned roughly in-plane, cells orientated non-tangentially to the cutting plane are present, with increasing proportions as one moves away from the epicardium. One needs to be careful, therefore, when comparing conduction speeds from different slices, and it may be necessary to perform histological analysis to assess underlying cell orientations. In addition, cells within a slice come from different transmural depth, as the cut is conducted in a true plane, not one that emulates an ‘onion shell’ of equidistant position relative to the ventricular surface (so cells in the centre of a slice will be from deeper transmural layers than those at the periphery). Thus, in spite of all efforts, slices will contain non-uniform structures, with varying degrees of cell alignment and coupling. These differences may create asymmetrical source-sink relations, which may reveal themselves, when pacing at different locations, in the form of differences in APD and conduction velocity.

Identification of an unbiased picture of APD distribution in tissue slices is therefore not possible with single point stimulation. Instead, multiple pacing points, and/or field stimulation, are called for. This is important for intervention testing, such as for drug application/washout studies, in particular if the pacing site is not controlled. If, for example, the pacing location varies before and after an intervention, changes in functional parameters that are due to source-sink heterogeneities could be mistakenly attributed to the effects of the intervention. Field stimulation would be more reliable for a before-and-after comparison, but the strength and orientation of the electric field relative to the tissue slice would also need to be controlled.

Data Analysis and Hypotheses for Further Investigation

Multi-parametric optical mapping generates large amounts of data per experiment, which makes data processing challenging. It is useful to employ automated or semi-automated routines, which analyse data sets, extract relevant information, save results, and generate maps and plots for user inspection. In this manuscript, we describe our approach to data processing and parameter extraction to characterise AP and CaT. Some of the parameters are standard (e.g. conduction velocity). Two new parameters, normalised area under AP curve (the AP area ratio) and AP skewness, were added into the routine to better characterise the shape of AP. Both may be useful for other researchers who wish to quantify and compare AP shapes ‘automatically’ without qualitative visual comparison.

We modified the method to evaluate APD by replacing the absolute upstroke peak value with the 95th percentile of the $V_m$ signal amplitude. This reduces the overly sensitive dependence of APD on the AP peak values. The most suitable percentile-value for this approach will depend on individual experimental conditions and AP shapes; in our analysis routine, this can be adjusted with no difficulty.
As fluorescence signals from tissue slices are weaker than those from perfused hearts or tissue wedges, it is important to process signal traces before parameter evaluation, in order to increase the reliability of the information extracted. As illustrated in the online supplemental movie, processing significantly reduces the impact of noise distortion on depolarisation and repolarisation phase detection. Multiple routines for processing optical mapping data have been published, including the one from Laughner et al. (42) for processing AP signals, mapped from whole hearts, with detailed explanation and comparison of spatial and temporary filters. Tian et al. (67) published their method to process single cell CaT signal which is based on fitting a bi-exponential curve to the decay phase of the CaT signal. We used an alternative curve-fitting based routine for processing AP signals. As shown in Figure 1, 1st order (for depolarisation) and 4th order polynomials (repolarisation) were chosen for curve-fitting routine. This works reasonably well for signals obtained from tissue slices (which tend to be more ‘noisy’ than signals from whole heart). For stronger signals with large signal-to-noise ratio, more computationally-efficient temporal filtering (IIR or FIR filtering) can be sufficient.

Averaging over a series of APs, obtained at the same pacing conditions without additional interventions, is a good way to improve signal-to-noise ratio. This was not used in our routine to retain the ability for analysing AP traces recorded during dynamic restitution protocols, and to explore beat-to-beat variability. The maximum upstroke velocity of the AP (dV/dt_max) is an important parameter, however limits in temporal resolution (~255 Hz for dual V_m/CaT measurement, ~510 Hz for single-parameter mapping) make estimation of dV/dt_max challenging. Over-laying a series of AP signals can be used to increase the number of data-points on the fast depolarisation phase. However in order to increase accuracy for dV/dt_max estimation, a measurement technique with higher temporal-resolution (e.g. fibre optics (45)) would be preferable.

Beside cardiac tissue slices, the data analysis routine presented in this manuscript will be applicable to other preparations, from single cell or cell cultures, to in silico simulated and native tissue AP recordings, and help to bridge the gap between experimental and computational modelling of the heart (59). Certain adjustments of threshold values (e.g. to identify upstroke timing) will be required when adopting it to other preparations.

Conclusions

Our data demonstrates that multi-parametric optical mapping, here of V_m and CaT, can be conducted on cardiac tissue slices of rabbit and guinea pig LV. We provide a method for semi-automated processing these data sets, and for extracting important descriptors of AP shape and conduction properties.

The use of cardiac tissue slices would benefit from standardisation, and we suggest adherence to the following points, or at least detailed documentation of equivalent aspects of the protocol:

- slices should be cut in an epicardium-tangential plane from electro-mechanically uncoupled tissue, using a high-precision vibratome with a low blade advance speed (~0.03 mm/s), sectioning tissue at no more than ~400 μm thickness;
- post-cutting, a recovery period in warm solution is needed for slices, cut in ice-cold solution, to reach steady-state electrophysiological properties; the recovery time may be species dependent (~35 min in guinea pig, ~ 60 min in rabbit);
- multiple-site point stimulation and field stimulation should be used for uncovering electrophysiological tissue heterogeneities, and any before/after investigations (such as drug testing) should control pacing location to exclude contributions of source-sink mismatches to the observed effects.

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**Figure Caption**

**Figure 1:** Flow chart of data processing and parameter extraction: A) raw $V_m$ signal trace; B) low-pass frequency filtering with a Butterworth filter (stop band 75 Hz, pass band 60 Hz) to estimate upstroke time point; C) preliminary identification of depolarisation (or upstroke) timing from the filtered trace; D) identification of AP maximum and $V_m$ baseline from the raw signal trace; calculation of 95th percentile and definition as maximal AP amplitude for further processing; E) correction for the identified upstroke time points; F) polynomial fitting through raw data points; G) linear correction for baseline drift; H) processed $V_m$ trace; I) estimating APD50 and APD80, based on defined maximal signal (as shown in D); J) calculating area under AP curve and the AP area ratio; K) calculating AP skewness; L) calculated conduction velocity. Grey regions in Figures 1I, 1J, 1K and 1L indicate tissue with weak or no signal ($((F_{max}-F_0)/F_0 \times 100\% <0.3\%)$. See ‘Data Analysis’ for detail.

**Figure 2:** Raw signal from dual $V_m$ and CaT mapping of a left ventricular tissue slice of rabbit (left) and guinea pig (right). A) Left panel: image of rabbit tissue slice, with red circle indicating the stimulation point and blue square indicating the area (3x3 pixels) used to plot $V_m$ and CaT traces (di-4-ANBDQPO and Rhod-2-AM; 2 Hz pacing). B) Normalised fluorescence intensity maps for $V_m$ and CaT from the sample in (A) at 7 time points, showing the progression of $V_m$ and CaT activation waves. C) Left panel: image of guinea pig tissue slice with red circle indicating the stimulation site and the blue square showing the region (3x3 pixels) used for raw $V_m$ and CaT traces (di-4-ANBDQBS with Cal-520-AM; 2 Hz pacing). D) Normalised fluorescence intensity maps of $V_m$ and CaT from the sample in (C) at 7 time points, showing the progression of $V_m$ and CaT activation waves.

**Figure 3:** Recovery in AP properties after cutting of tissue slices in ice-cold solution. A) Unfiltered $V_m$ traces, averaged over the whole slice, for (1) a guinea pig left ventricular slice at 2 min (blue) and 66 min (red) after removal from ice-cold BDM containing HEPES buffer. (2) a guinea pig left ventricular slice, 8 min (blue) and 73 min (red) after removal from ice-cold blebbistatin containing bicarbonate buffer, and (3) a rabbit left ventricular slice, 3 min (blue) and 151 min (red) after removal from ice-cold BDM containing HEPES buffer. B-1), B-2) and B-3) APD80 histograms of the slices above at several post-cutting time points during 2 Hz field stimulation. Time after cutting is colour coded.

**Figure 4:** Change in APD after cutting in ice-cold solution: Summary of normalised mean APD80 during 2 Hz pacing at different time points after cutting for guinea pig and rabbit slices (A: 8 slices from 5 guinea pigs; B: 7 slices from 4 rabbits; red curves show exponential fit).

**Figure 5:** Comparison of AP properties in matching tissue optically mapped in whole Langendorff-perfused guinea pig heart and in a tissue slices cut subsequently at body temperature (blebbistatin-containing bicarbonate buffer). A) Sub-epicardial APD80 map of the whole perfused LV, with the subsequently sectioned area outlined in red, and from the corresponding sub-epicardial tissue slice (slice 1) during field stimulation at 4 Hz. B) Histogram showing APD80 values for the LV area circled in red (in A) and from the corresponding sub-epicardial tissue slice, during field stimulation at 4 Hz. C) Averaged $V_m$ traces from the perfused whole heart LV (black) and the sub-epicardial tissue slice (red).

**Figure 6:** Influence of ice-cold temperature on AP properties: spatially averaged AP traces (averaged over the whole slice) measured at body temperature (79 min after initial cutting), and again at 5 and 18 min after a 1 h re-exposure to ice-cold solution.

**Figure 7:** APD80, conduction velocity and conduction speed maps of a rabbit slice, paced from two different locations (here at 1 Hz). A-1 and A-2) APD80 maps, with pacing sites indicated by black
circles. B-1 and B-2) conduction velocity (grey arrows) plotted on top of depolarisation time map. C-1 and C-2) conduction speed (in mm/ms). Grey areas indicate tissue with no significant fluorescent signal amplitude.

Figure 8: TEM and SEM images of rabbit slice tissue, fixed at different time points after cutting: A) TEM images 5 min after cutting, overview of intact cellular membrane structures (sarcolemma, mitochondria, T-tubules) and regular cross-striation (z-lines). B) enlarged view of sarcomeric protein structures with well-aligned isotropic and anisotropic bands. Magnification 7,500× in A, and 50,000× in B. C) – E) SEM images of tissue samples taken after 5 min; 1 h, and 4 h; respectively. All slices show cells with intact sarcolemma, preserved extracellular structures, and regular sarcomeric striation patterns.
**A)** captured raw signal with point interpolation
- raw trace

**B)** initial filtering (Butterworth filter) to filter out high frequencies to get upstroke time points
- filtered trace
- raw trace
- filtered trace

**C)** pick up upstroke points by looking at the first derivative of the trace
- end of upstroke time points

**D)** find baseline and define maximum signal for each AP
- draw histogram for each AP signal
- mean of this normal distribution = baseline
- 95 percentile is taken to be AP maximum
- draw histogram for each AP signal

**E)** correct upstroke time point calculation by fitting straight line through 1/3 to 2/3 way up
- rough upstroke

**F)** fit raw trace: linear line for upstroke phase, quartic curve for repolarisation phase
- raw trace
- fitted curve

**G)** linear correction for baseline drift
- fitted trace
- baseline drift correction reference line

**H)** processed data: baseline drift corrected and fitted V_m trace

**I)** measuring AP80 and APD50
- defined maximal signal
- 50% repolarisation threshold
- 80% repolarisation threshold

**J)** calculate normalised area under AP curve
- map of normalised area under AP curve
- Area 2
- Area 1

**K)** calculate rectangularity
- skewness of V_m signal
- skewness of V_m signal
- number of observations
- number of observations

**L)** map of depolarisation time
- map with depolarisation time
- estimated wavefront speed
A) Guinea pig

B) Rabbit

Normalized APD vs. Time after removal from ice-cold solution (min)

- Data
- Exponential fit