Alterations of field potentials in isotropic cardiomyocyte cell layers induced by multiple endogenous pacemakers under normal and hypothermal conditions

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Kienast R, Stöger M, Handler M, Hanser F, Baumgartner C. Alterations of field potentials in isotropic cardiomyocyte cell layers induced by multiple endogenous pacemakers under normal and hypothermal conditions. Am J Physiol Heart Circ Physiol 307: H1013–H1023, 2014. First published August 1, 2014; doi:10.1152/ajpheart.00097.2014.—The use of autonomous contracting randomly grown cardiomyocyte monolayers cultivated on microelectrode arrays (MEAs) represents an accepted experimental setting for preclinical experimental research in the field of cardiac electrophysiology. A dominant pacemaker forces a monolayer to adhere to a regular and synchronized contraction. Randomly distributed multiple pacemakers interfere with this dominant center, resulting in more or less frequent changes of propagation direction. This study aims to characterize the impact of changing propagation directions at single electrodes of the MEA on the four intrinsic parameters of registered field potentials (FPs) FPrise, FPMIN, FPre and CV, and conduction velocity (CV) under normal and hypothermal conditions. Primary cultures of chicken cardiomyocytes (n = 18) were plated directly onto MEAs and FPs were recorded in a temperature range between 37 and 29°C. The number and spatiotemporal distribution of biological and artificial pacemakers of each cell layer inside and outside of the MEA registration area were evaluated using an algorithm developed in-house. In almost every second myocardial cell layer, interfering autonomous pacemakers were detected at stable temperatures, showing random spatial distributions with similar beating rates. Additionally, a temperature-dependent change of the dominant pacemaker center was observed in n = 16 experiments. A significant spread-direction-dependent variation of CV, FPre, FPMIN, and FPrise up to 14% could be measured between different endogenous pacemakers. In conclusion, based on our results, disregarding the spatial origin of excitation may lead to misinterpretations and erroneous conclusions of FP parameters in the verification of research hypotheses in cellular electrophysiology.

microelectrode array; field potentials; hypothermia; spread-direction dependency; myocardial cells

THE USE OF RANDOMLY GROWN (“isotropic”) cell cultures represents an accepted in vitro model for preclinical experimental research to prove electrophysiological mechanisms in cardiac tissue. In particular, the cultivation of autonomous contracting cardiomyocyte cell cultures on microelectrode arrays (MEAs) permits both long-term and real-time monitoring of electrophysiological changes induced by external mechanical, chemical, or thermal stress (10) in a wide range of cardiovascular basic research applications (5, 12, 49) including pharmacological (drug or toxicity) testing (20, 33, 36, 38, 41, 43, 44, 54, 55). MEA technology serves as a high-throughput alternative to standard patch-clamp electrophysiology with relatively low costs (8, 40) where single electrodes of a MEA facilitate the registration of extracellular field potentials (FPs) at a multiplicity of defined points on the bottom of a cell culture. This enables the investigation of cellular electrical activity and electrophysiological alterations of entire myocardial cell complexes, including the spread of excitation and conduction velocity (CV) with high spatial and temporal resolution due to a linear relationship between relevant intrinsic features of FPs and action potentials (APs) (17). Furthermore, MEAs allow for conducting electrophysiological studies under hypothermal conditions (25, 46). A feasible approach for the investigation of electrophysiological changes of cardiomyocytes (e.g., hypothermia-induced changes or pharmacological stimuli) is the use of MEA technology for the registration of the FP for nonpatterned, randomly grown cell preparations of isolated cardiomyocytes. However, a randomly grown cell layer, referred to as an isotropic layer, is also comprised of randomly distributed pacemaker centers where a dominant center forces the layer to a synchronized regular contraction with definite direction of excitation propagation (18, 22). This process is influenced by other pacemaker centers (22), resulting in more or less frequent changes of propagation direction. The excitation propagation itself and, therefore, the CV as well as the morphology of FPs are influenced by many factors such as type, amount, conductance and distribution of gap junctions (2, 27, 40, 50), ratio between myofibroblasts and cardiomyocytes (13, 28), cell size (27, 50), orientation of the cardiomyocytes (7, 14, 27), intercellular clefts (13), and cell density (34). In a randomly grown (isotropic) cell layer all of these electrophysiological parameters can be diversified throughout the substructural cell organization (Fig. 1), which may lead to slightly different registration results at different electrode positions in the MEA. As a consequence we introduced the term “quasi-isotropic” for a randomly grown cell layer of embryonic chicken cardiomyocytes, which is the study material featured in this work. In addition to these spatial changes in registered signals, local (microscopic) anisotropies in randomly grown cell layers may lead to fluctuations in registered signals at single electrodes induced by multiple active pacemaker centers and associated propagation direction changes (22, 47).

To demonstrate the problem of different registration results, Fig. 2 exemplarily illustrates physiologically unexpected jumps of rise time of registered field potentials (FPrise) caused by switches of the active pacemaker at 35°C, recorded at a single electrode in the MEA. FPrise correlates linearly with the AP rise time and is an accepted indicator for changes in the AP
ALTERATIONS OF FIELD POTENTIALS IN CARDIOMYOCYTES

upstroke velocity (17). However, this observation can lead to misinterpretations in signal analysis when, for example, studying the characteristics of upstroke velocity at different temperatures or after pharmacological stimuli as also hypothesized in the work of Sommerhage et al. (47).

Therefore, the investigation of electrophysiological alterations in quasi-isotropic cell cultures (including multiple pacemaker centers) under normal and/or hypothermal conditions leads to the following scientific question:

Is there evidence of excitation direction changes and alterations of extracellularly recorded FPs in quasi-isotropic myocardial cell layers registered at single electrodes of the MEA under normal and hypothermal conditions?

To tackle this question detailed information about spatial distribution and temporal activation of pacemaker centers and related changes of wavefront propagation direction is needed to study the impact of the spatial origin of excitation on selected intrinsic FP features under hypothermal conditions. One approach is to use artificial stimulation with well-defined stimulation parameters for time and position. Compared with autonomous pacemakers, artificial pacing may, however, lead to unexpected changes of electrophysiological parameters such as the CV (40). To investigate these mechanisms a new algorithm was developed to compute spatial and temporal distribution of biological and artificial excitation sources located inside and outside the MEA registration area. Through the use of this approach we are able to demonstrate that CVs and intrinsic parameters of FPs in quasi-isotropic myocardial cell preparations depend on the direction of excitation propagation. Neglecting these changes in excitation propagation under normal, mild, and moderate hypothermic temperatures may result in misinterpretations of microscopic cardiac determinants.

METHODS

Cell cultivation. In this study we analyzed intrinsic parameters of FPs for n = 18 primary cultures of chicken cardiomyocytes. As previously described in Ref. 25, the hearts of 12-day-old chicken embryos were extracted and the ventricles were separated from the atrium. The isolated ventricles were minced and digested with 0.05% trypsin. One drop of the obtained cell suspension was plated onto the fibronectin-coated MEA covering the electrode grid located in the center of a Petri dish. One to two minutes thereafter the Petri dish was carefully filled with 1.5 ml of culture medium. One milliliter of medium was renewed daily. After 3–4 days of incubation, the cells formed a firmly attached and spontaneous beating monolayer covering the whole electrode area that was used for MEA recordings (Fig. 3). After registration of the FPs under hypothermal conditions, each cell preparation was ineligible for further measurements and discarded.

MEA recording. Eighteen planar MEAs (Multi Channel Systems, Reutlingen, Germany) with three different electrode configurations and an integrated reference electrode placed on the bottom of the

Fig. 1. A schematic illustration of a randomly grown “quasi-isotropic” myocardial cell layer with multiple active pacemaker centers outside the microelectrode arrays (MEA) registration area. At first appearance the morphological structure of the cell layer seems to be homogeneous. Considering the substructural cell organization (see zoomed areas) randomly grown cardiomyocytes formed locally an anisotropic but not predictable structure (in terms of cell density and orientation, cell size, distribution of gap junctions, and their conductance). See also Fig. 3.

Fig. 2. A: spatial distribution of pacemakers in the myocardial cell layer where number 2 indicates an artificial stimulus. B: boxplot of the spread-dependent field potential rise (FP_{rise}) time for the 3 different pacemaker (2 biological and 1 artificial) centers in a single cell layer at 35°C measured at a single electrode. C: yields the same data as in B over time. Physiologically unexpected jumps in data caused by alternations of the pacemaker centers are evident. The rectangle in A indicates the active MEA registration area.

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culture dish was used for extracellular recording (Table 1). The culture dish with built-in MEA was fixed on a USB-MEA60-Inv-System amplifier and recording system (Multi Channel Systems). Data were registered simultaneously from all 60 channels with a sampling frequency of 20 kHz each and a bandwidth of 1–3 kHz. Due to damaged electrodes, or poor contact between the cardiomycyte monolayer and individual electrodes the signal quality was partly impaired in some of the available electrodes. Note that one reference electrode and partially two stimulus electrodes decrease the number of available registration channels. On average signals from 53(6) electrodes per cell preparation could be used for the analysis, which resulted in signal data from \( n = 950 \) electrodes over all experiments. To enable measurements under hypothermical conditions the system was extended by an adjustable heating and cooling device developed in-house, as recently described in (25). This device can adjust the temperature of the myocardial cell layer in the range from \( T = 10°C \) to \( T = 40°C \). All measurements started at \( T = 37°C \). The temperature was reduced in steps of \( 2°C \) every 5(6) min and FPs were registered. Figure 4 illustrates exemplarily registered signals at different temperatures of 37, 35, 33, 31, and 29°C. For artificial pacing, two recording electrodes in the middle right center of the MEA were used by applying biphasic square pulses with a stimulus generator (STG 4004; Multi channel Systems) in \( n = 13 \) cell layers. These artificial stimuli were set at different temperatures with an amplitude of \( \pm 1.5 \) V and a pulse width of 400 \( \mu \)s each. Each pulse was repeated 20 times with a higher frequency than the dominant biological pacemaker’s frequency (pulse width of 400 \( \mu \)s). Each pulse was repeated 20 times with a higher frequency than the dominant biological pacemaker’s frequency (pulse width of 400 \( \mu \)s).

**Detection of pacemaker centers.** To estimate the spatiotemporal distribution of active biological pacemakers in a myocardial cell preparation a new algorithm was used as recently described in Ref. 25. This algorithm computes the spatiotemporal distribution of all active pacemaker centers in and outside the MEA registration area and allows for an assignment of each individually registered wavefront to an active pacemaker center. This information permits a direction-dependent separation of FPs for further analysis. A brief description of the algorithm can be found in the APPENDIX (see Pacemaker detection algorithm).

**Spread-dependent features.** In a quasi-isotropic cultured cell layer structural and morphological inhomogeneities may cause alterations in FPs depending on the origin of excitation. On the basis of the work by Halbach et al. (17) the following intrinsic features of FPs were studied to verify our hypothesis (Fig. 5):

1. The first positive peak of the FP (FP\(_{\text{pre}}\)), which corresponds to a contribution of neighboring, previously excited, tissue.
2. The first negative peak of the FP (FP\(_{\text{MIN}}\)) depends critically on Na\(^+\) current.
3. The duration of the FP (FP\(_{\text{dur}}\)), which correlates to the AP duration and is defined as the time between FP\(_{\text{MIN}}\) and FP\(_{\text{MAX}}\).
4. The FP rise time (FP\(_{\text{rise}}\)), which correlates to the AP upstroke time and is defined as the time span between decline of the voltage from baseline to FP\(_{\text{MIN}}\) (between 10 and 90%).

The morphology of the depolarization wave FP\(_{\text{MAX}}\) is slightly different compared with the study of Halbach et al. (17) using adult black-6 mice. In our experiments with chicken cardiomycocytes at \( 37°C \), we could observe a small depolarization wave roughly 210 ms after FP\(_{\text{MIN}}\) with a mean amplitude of 12 \( \mu \)V. Analogous time parameters for repolarization, also measured for chicken cardiomycocytes, were described by Krogh-Madsen et al. (31). This small depolarization wave is apparent if the registered signals yield a stable baseline with a small noise component. However, the poor signal-to-noise ratio relative to FP\(_{\text{MAX}}\) somewhat limited the detection accuracy of the depolarization wave and consequently the FP\(_{\text{dur}}\) time in our experiments.

In addition to the previously described FP features, differences of CV in single electrodes were determined depending on the origin of excitation. Analogous to previously introduced methods (4, 37), CV fields registered from in vitro experiments were calculated from the two-dimensional spatial gradient of the waveform arrival time \( T(x,y) \). The gradient of a single wavefront moving across the MEA was calculated numerically, exactly at the position of the electrodes. This implies that the local coordinates \( x \) and \( y \) are held constant and only activation time \( t \) changes. Therefore, the velocity vector \( v = [d/dt, dy/dt] \) of each single electrode can be simply computed by inverting the elements of the gradient \( \text{grad}T = ([\partial T/\partial x, \partial T/\partial y]) \) to obtain the velocity vector field \( V(x,y,t) \). It is well known that decreasing temperature has a strong impact on cardiac electrophysiology such as prolonged depolarization time and decreased CV and firing rate of pacemaker cells. However, in this study we investigated the influence of spread direction of multiple endogenous pacemakers on intrinsic FP features and CV by analyzing FP morphologies at stable temperature levels of 37, 35, 33, 31, and 29°C to exclude side effects during temperature changes.

**Data analysis.** The data were analyzed offline with an in-house developed software tool based on MATLAB (The Mathworks, Natick, MA) to detect and characterize FP features and CV. To statistically evaluate direction-dependent effects of the four FP features FP\(_{\text{rise}}\), FP\(_{\text{MIN}}\), FP\(_{\text{pre}}\), and FP\(_{\text{dur}}\) and CV at single electrodes of the MEA, first a Shapiro-Wilk test was applied to test whether the data follows a normal distribution (\( P < 0.05 \)). Since most of the data is not normally distributed, a Kruskal-Wallis one-way analysis was employed to compare the values of the respective feature for two or more different active pacemakers at different temperature levels for statistical significance (nonparametric multiple class testing). Differences were considered statistically significant for \( P < 0.01 \) using Bonferroni correction for multiple Kruskal-Wallis one-way testing. Due to the

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**Table 1. Electrode configurations for FP registrations**

<table>
<thead>
<tr>
<th>Electrode Diameter, ( \mu m )</th>
<th>Electrode Material</th>
<th>Interelectrode Distance, ( \mu m )</th>
<th>Electrode Layout</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>TiN</td>
<td>200</td>
<td>grid, 8 x 8</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>TiN</td>
<td>200</td>
<td>grid, 8 x 8</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>Gold</td>
<td>700</td>
<td>grid, 8 x 8</td>
<td>3</td>
</tr>
</tbody>
</table>

FP, field potential; TiN, titanium nitride; \( n \), number of performed experimental studies with the same electrode configuration.
unpredictable number and various beat intervals of active pacemaker centers, the number of Kruskal-Wallis tests differs between the individual temperature levels. However, to obtain a comparable representation, results are shown as bar charts normalized to 100% and the number of the performed Kruskal-Wallis tests is denoted in the respective bar as additional information. Due to altered morphology of the different myocardial cell preparation, distinct electrode-cell-coupling, and temperature, absolute values of investigated features vary. To illustrate the impact of various propagation directions, relative changes of FP features between the pacemaker center with the lowest and highest values are calculated. Because of predominantly left skewed data distributions, these changes are denoted by the median and the median absolute deviation (MAD).

RESULTS

Absolute values of intrinsic FP features and CV. Table 2 shows mean values of CV and investigated intrinsic FP features determined from our experimental data. The registered signal amplitude of FPpre and FPMIN depends on the selected electrode array. All other features show no significant differences between the used electrode configurations. In addition, well known hypothermia-induced effects such as a decrease in CV or an increase in AP duration (FPdur) can be observed.

Pacemaker centers. As described in Refs. 10 and 21, a myocardial cell culture forms weakly coupled islets of cell aggregates with individual active pacemakers in the first days of incubation. After 3–4 days, these islets merge to a continuous cell layer and a dominant center emerges. This center forces the layer to a synchronized, stable, and regular contraction, more or less interfering with other randomly distributed pacemakers where the contraction frequency depends on the initial seeding density and the age of the study animal. The phenomenon of multiple pacemaker centers in a culture has been thoroughly discussed for embryonic stem cells of line D3 (mouse) (22) for primary cultures of rat ventricular myocytes (15, 47) and murine atrial cardiomyocyte-derived cell line HL-1 (30). In our experimental data, multiple autonomous (biological) pacemaker centers were detected in almost every second myocardial cell layer at different stable temperature levels of 37, 35, 33, 31, and 29°C (Fig. 6). As also described by Feld et al. (15) and confirmed by our observations, in most cases pacemaker centers formed outside the MEA registration area. Table 3 gives detailed information about the frequency of all active pacemaker centers and their average counts with standard deviations in mild and moderate hypothermia. The beating rates of active pacemakers show stable frequencies of about 0.5 Hz (30 beats/min) at 29°C to ~1.5 Hz (90 beats/min) at 37°C (Fig. 7A). The beating rates of interfering pacemaker centers are in a similar range where the frequency variation is in most of the experiments <0.1 Hz (6 beats/min; Fig. 7B). In addition to the interference of the dominant pacemaker center by other pacemakers at stable temperatures, we also observed a temperature-dependent alteration of propagation direction that led to changes of the dominant pacemaker region in the cell layer during cooling in almost all analyzed myocardial cell cultures [n = 16 of total n = 18; a link to movies, visualizing changes in excitation direction is provided in the appendix (see links to raw data and movies)]. However, the number of active pacemaker centers at the prevailing temperature and the position of the dominant center differ from experiment to experiment. In our cell preparations 4.0 (SD 1.9) centers on average...
were detected in the considered temperature range from 37 to 29°C. Figure 8 illustrates time series experiments of active pacemaker centers and their spatial distributions for two cell layers. Temperature-induced changes of the dominant pacemaker center can be observed in both cell layers.

Conduction velocity. Figure 9A depicts a boxplot of calculated CV of two autonomous pacemaker centers from the same electrode at different temperatures. The well-known effect of CV reduction with decreasing temperature is evident. Statistical analysis of CV for all experimental datasets using a Kruskal-Wallis test, however, yields a significant correlation between CV and propagation direction at stable temperatures (Fig. 10A) with a median difference of 6% (MAD = 4) between different pacemakers.

$F_{\text{Prise}}$. The spread dependency of $F_{\text{Prise}}$ time measured at single electrodes could be experimentally confirmed for different temperatures. Figure 9B shows a boxplot demonstrating a correlation of $F_{\text{Prise}}$ and CV for active pacemaker centers at the same electrode position. The bar graph in Fig. 10B summarizes the results of multiple testing for all experiments in the given temperature range from 37 to 29°C. The median change of $F_{\text{Prise}}$ between different propagation directions of multiple pacemakers is $\sim 14\%$ (MAD = 8).

Furthermore, Halbach et al. (17) and Banach et al. (3) observed a distance-dependent behavior of $F_{\text{Prise}}$ with respect to the location of a given pacemaker center. In this study we were not able to confirm a significant relationship between $F_{\text{Prise}}$ and the distance to the pacemaker centers (see Distance-dependent behavior of $F_{\text{Prise}}$ in the Appendix).

$F_{\text{PMIN}}$. As known, there is a significant relationship between $F_{\text{PMIN}}$ and $Na^+$ current (17). Again Kruskal-Wallis testing was performed (Fig. 10C), confirming a spread-direction dependency of the parameter $F_{\text{PMIN}}$ with a median change of $\sim 13\%$ (MAD = 8) as shown exemplarily in Fig. 9C for a single experiment.

$F_{\text{Pre}}$. Halbach et al. (17) described $F_{\text{Pre}}$ as a contribution of neighboring, previously excited tissue. Interestingly, based on the correlation between $F_{\text{PMIN}}$ amplitude and temperature (see $F_{\text{PMIN}}$ in RESULTS) our experiments also yielded a decrease in the amplitude of $F_{\text{Pre}}$ with dropping temperatures. In addition, we observed a propagation direction dependency of $F_{\text{Pre}}$ that could be statistically confirmed by multiple testing (Figs. 9D and 10D) for all considered temperature levels. The determined $F_{\text{Pre}}$ values vary by $\sim 13\%$ (MAD = 8) between different excitation directions.

$F_{\text{DUR}}$. $F_{\text{Dur}}$ is defined by the time between $F_{\text{PMIN}}$ and $F_{\text{MAX}}$ and shows a relationship to AP duration (17). Similar to the repolarization time of chicken cardiomyocytes as described by Krogh-Madsen et al. (31), $F_{\text{MAX}}$ was measured $\sim 240$ ms after $F_{\text{MIN}}$ at 37°C. After visual inspection of our experimental data and according to the literature (16, 26), $F_{\text{Dur}}$ is estimated to be in the range between 300 and 500 ms at the lowest considered temperature level of 29°C. Based on the fact that AP duration increases with decreasing temperatures (1, 26), $F_{\text{Dur}}$ can, therefore, be expected in the range of 240 to 500 ms. Due to the poor signal-to-noise ratio relative to $F_{\text{MAX}}$, the detection accuracy of $F_{\text{Dur}}$ is, however, somewhat limited. This leads to some false detections of $F_{\text{Dur}}$ outside the expected physiological range. As a consequence, $F_{\text{Dur}}$ data beyond the range of 100 to 800 ms were removed before Kruskal-Wallis testing. Nevertheless, a direction dependency of $F_{\text{Dur}}$ could not be obviously confirmed at the single electrodes (Figs. 9E and 10E). The averaged difference between varying excitation directions is, however, not $> 1\%$.

Impact of artificial stimuli on CV and intrinsic FP parameters. As aforementioned, in $n = 13$ myocardial cell layers an artificial stimulus was set in the middle right center of the MEA. We observed a stronger impact on the FP morphology and CV for artificial stimuli compared with biologically

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>CV, cm/s</th>
<th>$F_{\text{Prise}}$, ms</th>
<th>$F_{\text{Pre}}$, mV</th>
<th>$F_{\text{PMIN}}$, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>20.4</td>
<td>0.194</td>
<td>-0.54</td>
<td>348</td>
</tr>
<tr>
<td>31</td>
<td>22.3</td>
<td>0.153</td>
<td>-0.56</td>
<td>312</td>
</tr>
<tr>
<td>33</td>
<td>24.0</td>
<td>0.155</td>
<td>-0.58</td>
<td>279</td>
</tr>
<tr>
<td>35</td>
<td>26.0</td>
<td>0.137</td>
<td>-0.53</td>
<td>246</td>
</tr>
<tr>
<td>37</td>
<td>27.8</td>
<td>0.138</td>
<td>-0.64</td>
<td>240</td>
</tr>
</tbody>
</table>

Table 3. Myocardial cell cultures with more than 1 active pacemaker center at temperatures of 29, 31, 33, 35, and 37°C

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>$n \geq 2$ Active Pacemakers/Total Number of Cell Layers</th>
<th>No. of Active Pacemakers (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>7/12</td>
<td>2.29 (SD 0.49)</td>
</tr>
<tr>
<td>31</td>
<td>9/17</td>
<td>2.44 (SD 0.53)</td>
</tr>
<tr>
<td>33</td>
<td>6/18</td>
<td>2.17 (SD 0.41)</td>
</tr>
<tr>
<td>35</td>
<td>9/18</td>
<td>3.00 (SD 0.87)</td>
</tr>
<tr>
<td>37</td>
<td>10/18</td>
<td>2.70 (SD 0.95)</td>
</tr>
</tbody>
</table>

In the majority of cell layers 2 or 3 centers were active.
autonomous pacemakers (see Impact of artificial stimuli on CV and intrinsic FP parameters in the Appendix).

**DISCUSSION**

Recording FPs using MEAs is a common tool for investigating various scientific questions in the field of cardiac electrocardiography, for example, in in vitro pharmacological stimulation and testing. The correlation between time and shape features of the AP and FP invites us to draw conclusions regarding the electrophysiological mechanisms on cardiomyocyte cell layers during cooling. Excitation-dependent features such as CV are, however, difficult to detect from recorded signals in unorganized homogeneous cardiac monolayers (10, 40). In addition, varying directions of propagation due to alternating active pacemaker centers hamper the analysis and interpretation of these parameters. Moreover, it is not entirely known how these biological variations affect intrinsic features of locally registered FPs at single electrodes of the MEA. When MEA technology, used to register FPs under hypothermic conditions, is combined with our in-house developed algorithm for pacemaker detection, analysis of local and global intrinsic FP parameters in a monolayer with multiple pacemaker centers is possible for each separate pacemaker center. Utilizing this approach we are able to demonstrate a relative change of up to 14% for CV and intrinsic FP parameters (FP\textsubscript{rise}, FP\textsubscript{MIN}, and FP\textsubscript{pre}) caused by alternating propagation directions at different temperatures and individual electrodes of the MEA regardless of the used electrode configurations (Table 1).

**Pacemaker centers.** Our experimental data have demonstrated a frequent occurrence of endogenous multiple pacemaker centers in quasi-isotropic grown cell cultures at stable temperatures. Based on this observation we investigated the impact of alternating excitation directions on intrinsic FP features and identified significant differences. However, different beating rates lead to electrophysiological changes such as alteration of AP duration or upstroke velocity (29, 42, 51). To exclude beating-rate-induced effects, an analysis of the changes of the beating rate among competitive endogenous active pacemakers was performed showing, interestingly, almost similar frequencies. We conclude that an autonomous random changeover procedure between dominant and interfering pacemakers at stable temperature levels leads to a change in excitation direction at almost stable beating rates. Therefore, the observed alterations of intrinsic FP features are caused by excitation direction changes so that electrophysiological effects of different beating rates can be neglected.

In addition to competitive pacemaker centers at stable temperatures, endogenous pacemakers respond differently to a temperature reduction by changing their spatial activation patterns. This also leads to a change of direction in dominant wave-front-propagation patterns. The question is: when does a pacemaker achieve the activation threshold to synchronize the surrounding tissue? Thereby two main factors play a crucial role (24):

1) The coupling conductance between the pacemaker center and surrounding cell structure.

2) The spatial extension of the pacemaker center (number of cells) and their intercellular coupling (morphology).

Since the gap junctions are responsible for the intercellular electrical coupling, the number and electrical conductance of single gap junctions define the conductivity between single cells (45). If the conductivity is high enough, an existing pacemaker center in a cell layer can serve as a starting point for an excitation (52). If more than one pacemaker center fulfills this criterion, the center with the highest frequency drives the cellular layer (19, 32).

According to these physiological mechanisms we discuss two possible scenarios for a hypothermia induced pacemaker change:

1) The active pacemaker center fulfills the criteria to drive the cellular layer and has the highest frequency. However, compared with another center it has a reduced number of gap junctions connecting the center to the surrounding cells. Since the gap junction conductivity decreases with falling temperature (6, 9), the conductivity through the reduced number of gap junctions of the active pacemaker reaches a critical limit during cooling and therefore loses its ability to stimulate the cellular layer. Due to the higher number of intercellular connections, another center does not fall below this critical limit and becomes the active center.

2) Hypothermia induces a decreased spontaneous depolarization of pacemaker cells, which results in a lower frequency of the pacemaker center (19, 35, 53). If during cooling another endogenous center exhibits a higher rate than the currently dominant center, this center becomes dominant.
In our case of randomly grown cell layers (see Fig. 1), it is not obvious how many potential pacemaker centers are inherent in the cell culture, how distinct their electrical coupling is, and how sensitive the beating rate of each center is when responding to hypothermic conditions. Additionally, the fact that in most cases the pacemaker regions are outside the MEA registration area hampered us to study the physiological mechanisms of hypothermia-induced pacemaker activation. Because

![Figure 8](image_url)

Fig. 8. Temporal activation patterns of active pacemaker centers during cooling in the temperature range of 37 to 29°C. A: id 1 and id 3 are biological pacemaker centers while id 2 indicates an artificial stimulus. In the temperature range from 37 to 35°C the dominant pacemaker center 1 interferes with center 3. A change to dominant center 3 can be observed between 35 and 33°C. F: id 1 and id 3 to 5 are biologically autonomous pacemaker centers while id 2 indicates an artificial stimulus. Three temperature-induced changes of the dominant pacemaker center are observed. In both diagrams A and F, id 0 indicates wavefront propagation centers which could not be assigned by the detection algorithm. B and G: spatial distribution of pacemakers in the myocardial cell layer of the corresponding experiment (A and F). The square marks the MEA registration area. C, D, and E: isochronal maps calculated in the MEA registration area for all 3 active pacemakers corresponding to the experiment in A at 35°C. The numbers on the isochronal lines denote the wavefront propagation in millisecond. The MEA covers an area of 1.4 × 1.4 mm.

![Figure 9](image_url)

Fig. 9. A–E: selected boxplots of spread-dependent FP features for two different autonomous pacemaker centers in a single cell layer at 31 and 39°C. It is obvious that measured values of each feature, except for FPdur, differ as a function of location of the pacemaker center and temperature.
of the complexity of such highly random mechanisms and the lack of measuring pacemaker signals directly, we are not able to establish a predictive model on our experimental results for estimating the temperature-dependent alteration of pacemaker centers in time and space.

Conduction velocity. CV changes significantly with different orientations of myocardial cells as well as varying conductances and distributions of gap junctions. With the proposed method, relying on a spatially constant electrode position to calculate the velocity, the same cell layer segments can be measured repeatedly. This allows for a spatial and temporal comparison of the velocity in the experimental data. We could confirm a spread-direction dependency in randomly grown multicellular preparations of cardiac myocytes under normal and hypothermal conditions. In particular, the effect of excitation directions has a significant impact on intrinsic electrophysiological parameters of registered FPs in randomly grown multicellular preparations of cardiac myocytes under normal and hypothermal conditions. In particular, the effect of excitation directions has a significant impact on intrinsic electrophysiological parameters of registered FPs in randomly grown multicellular preparations of cardiac myocytes under normal and hypothermal conditions.

FP_{rise}. As shown, the detection of FP_{rise} at single points in a quasi-isotropic myocardial cell layer is highly sensitive to the direction of propagation. Due to the linear relationship between FP_{rise} and AP rise time, the observed changes indicate a possible direction-dependent alteration in the upstroke velocity of AP.

FP_{MIN} and FP_{pre}. The two prominent peaks FP_{MIN} and FP_{pre} in a FP are caused by the excitation of the local and surrounding tissue of the observed electrode, respectively, where FP_{MIN} precedes in time with the maximum upstroke velocity of the AP and correlates to the Na⁺ current (17). Our experimental data showed that the amplitudes of these parameters also exhibit direction-dependent characteristics at single points inside the cell layer.

FP_{dur}. FP_{dur} is an important indicator for the repolarization of myocardial tissue. It is known that hypothermia induces a prolongation of repolarization time that is coupled with a prolongation of the AP (1, 26). This effect is also evident in our experimental data. However, for the investigation of hypothermal-induced changes of FPs, it is important to know whether FP_{dur} varies with different propagation directions. Statistical testing, taking into account FP_{dur} for single electrodes, revealed in ~60% of experiments no significant difference between FP_{dur} of the single pacemaker centers. However, because of the small magnitudes of the repolarization wave (FP_{MAX}), the detection accuracy for local FP_{dur} was limited.

Conclusion. In this study we demonstrated that alternating excitation directions has a significant impact on intrinsic electrophysiological parameters of registered FPs in randomly grown multicellular preparations of cardiac myocytes under normal and hypothermal conditions. In particular, the effect of spread-direction dependency could be experimentally verified and validated with established direction-dependent features such as CV or FP_{rise} using MEA technology. In addition, we observed a spread dependency of FP spike amplitudes (FP_{MIN} and FP_{pre}). Furthermore, the observation of spread dependency at a stable temperature implies a similar effect also at altered

Fig. 10. Multiple group testing of intrinsic FP features for single electrodes at different temperature levels. The null hypothesis of equal conduction velocity (CV; A), FP_{rise} (B), FP_{MIN} (C), and FP_{pre} (D) at different pacemaker centers has to be rejected with a P value of 0.01 in almost all experiments. However, a significant direction dependency of FP_{dur} (E) could not be confirmed. n Indicates the number of performed Kruskal-Wallis one-way tests; P < 0.01 and P > 0.01.
temperature levels. This is essential in the analysis of hypothermal alterations since we demonstrated that cooling down a quasi-isotropic myocardial cell culture, and the fact that myocytes are highly sensitive to temperature changes (11), result in a spatial change of the dominant pacemaker center in a quasi-isotropic cell layer in almost all experiments. This also leads to a change of propagation direction as demonstrated. Bypassing this spread-dependent effect by application of artificial stimuli, however, has two main disadvantages: first, lack of beating rate information of the biological pacemakers, and secondly, the risk of possible alterations of electrophysiological parameters by artificial stimuli. Therefore, we can conclude that changes in propagation direction coming from alternating endogenous biological pacemakers need to be considered in the analysis and interpretation of electrophysiological mechanisms of non-patterned, randomly grown cell preparations of isolated cardiomyocytes at different temperature levels. Disregarding these observations can lead to misinterpretations and erroneous conclusions in the verification of research hypotheses in molecular and cellular electrophysiology.

APPENDIX

Pacemaker detection algorithm.

To distinguish registered wavefronts of different propagation direction, the algorithm computes the spatiotemporal distribution of all active pacemaker centers in and outside of the MEA registration area using a two-step approach (25):

1) Calculating the starting point of excitation for each individual registered wavefront based on the negative two-dimensional spatial gradient of the wavefront’s arrival time within the area of the electrode array.

2) Identifying local clusters of active pacemaker centers in the cell culture using the information of the first step. Applying this paradigm assigns each individual registered wavefront to an active pacemaker center. This information permits a direction-dependent separation of propagation features for further analysis. Figure A1 shows superimposed isochronal maps of all single beats (n = 949) of a selected cell layer separated by the differentiated excitation directions.

Distance-dependent behavior of \( F_{\text{Prise}} \).

To confirm the distance-dependent behavior of \( F_{\text{Prise}} \) observed by Halbach et al. (17), a correlation analysis was performed. For each temperature level and each pacemaker center, the Pearson correlation coefficient \( r \) was calculated where the distance between pacemaker and electrodes was correlated with the median value of \( F_{\text{Prise}} \) for each single electrode. The box plots in Fig. A2 show the calculated \( r \) values for the different temperatures. It is clear that we were not able to confirm a significant relationship between \( F_{\text{Prise}} \) and the distance to the pacemaker centers (Fig. A2A). The same analysis was performed solely for the artificial stimuli (without autonomous pacemaker centers), showing a similar result, however, with a slight negative correlation coefficient of about \( -0.2 \) (Fig. A2B).

Interestingly, Spach et al. (48) also found a correlation between AP upstroke and the time interval between \( F_{\text{Pre}} \) and \( F_{\text{PMN}} \). This observation could be confirmed indirectly by an \( F_{\text{Prise}} \) increase with decreasing temperatures \( r = 0.87 (SD 0.25), P < 0.01 \). Therefore, the time interval \( [F_{\text{Pre}}, F_{\text{PMN}}] \) is also expected to increase analogously to \( F_{\text{Prise}} \) and is a suitable measure that can be used alternatively to \( F_{\text{Prise}} \) for the investigation of temperature induced changes of the myocardial AP. A further advantage is, if signals show strong baseline drifts, that the \( F_{\text{Pre}}-F_{\text{PMN}} \) interval compared with \( F_{\text{Prise}} \) is almost independent of baseline variations.

Impact of artificial stimuli on CV and intrinsic FP parameters.

It is known that rapid electrical stimulation (RES) causes alterations in myocardial gap junctions (modulated by the expression of the gene Connexin 43), which resulted in an increase of CV after ~60 min (23, 39, 40). In contrast to the reported stimulation frequency of RES (~3 Hz) our stimulation frequency was in the range of 0.7 to 2 Hz with a maximal duration of 30 s per stimulation sequence. However, visual inspection of the data reveals a remarkably strong variation of intrinsic FP parameters, caused by biological and artificial stimuli (for example, see \( F_{\text{Prise}} \)).
in Fig. 2, B and C). To investigate the influence of the applied stimulus, we hypothesized as follows.

An artificial stimulus has a stronger impact on the FP morphology and CV than biologically autonomous pacemakers, and therefore, the relative median changes of these parameters between biological pacemakers and artificial stimulus are significantly different than those found solely among the natural pacemakers.

To prove this hypothesis we selected only those data from our measurements where, at a stable temperature, at least two biological pacemakers and the artificial stimulus were active. Based on these data we repeated previously described analyses for all intrinsic FP features. In a first step, the artificial stimuli were excluded, considering only biological pacemakers. In the second step, the artificial stimulus was included to compare the common effects of biological and artificial stimuli. The results revealed a slight, but significant, difference (P < 0.01, Mann-Whitney U-test) for all investigated features (Fig. A3). In detail, FP_{rise} and FP_{MIN} increased in the presence of an artificial stimulus, where CV, FP_{pre}, and FP_{dur} decreased compared with the natural pacemakers. Therefore we could confirm that artificial stimuli have a stronger impact on the FP morphology and CV. Interestingly, compared with the aforementioned, insignificant relationship between repolarization time (FP_{dur}) and direction dependency of different biological pacemakers (see FP_{dur} in RESULTS), we found a significant difference between biological and artificial stimuli (P < 0.01) (Fig. A4). To conclude, the impact is small, but significant, in all investigated FP features and CV. However, to validate these observations, in particular if these changes are caused by electrophysiological variations between biological and artificial stimuli or merely due to an increase in beating rate, additional comprehensive experiments are needed.

**Links to raw data and movies.**

Selected raw data in MATLAB format (The Mathworks, Natick, MA) and movies, illustrating changes in excitation direction are available at: http://www.umit.at/page.cfm?path=departments/technik/iebe/tools/propagation_direction_dependent_fps&switchLocale=en_US

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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