Multi-Imaging Method to Assay the Contractile Mechanical Output of Micropatterned Human iPSC-Derived Cardiac Myocytes

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Rationale: During each beat, cardiac myocytes (CMs) generate the mechanical output necessary for heart function through contractile mechanisms that involve shortening of sarcomeres along myofibrils. Human-induced pluripotent stem cells (hiPSCs) can be differentiated into CMs (hiPSC-CMs) that model cardiac contractile mechanical output more robustly when micropatterned into physiological shapes. Quantifying the mechanical output of these cells enables us to assay cardiac activity in a dish.

Objective: We sought to develop a computational platform that integrates analytic approaches to quantify the mechanical output of single micropatterned hiPSC-CMs from microscopy videos.

Methods and Results: We micropatterned single hiPSC-CMs on deformable polyacrylamide substrates containing fluorescent microbeads. We acquired videos of single beating cells, of microbead displacement during contractions, and of fluorescently labeled myofibrils. These videos were independently analyzed to obtain parameters that capture the mechanical output of the imaged single cells. We also developed novel methods to quantify sarcomere length from videos of moving myofibrils and to analyze loss of synchronicity of beating in cells with contractile defects. We tested this computational platform by detecting variations in mechanical output induced by drugs and in cells expressing low levels of myosin-binding protein C.

Conclusions: Our method can measure the cardiac function of single micropatterned hiPSC-CMs and determine contractile parameters that can be used to elucidate mechanisms that underlie variations in CM function. This platform will be amenable to future studies of the effects of mutations and drugs on cardiac function. (Circ Res. 2017;120:1572-1583. DOI: 10.1161/CIRCRESAHA.116.310363.)

Key Words: cardiac myocyte ■ contractility ■ sarcomere length ■ single cell ■ stem cell

Cardiac myocytes (CMs) are the muscle cells of the myocardium that beat to generate the mechanical output required for heart function. The mechanical output of CMs originates from the shortening of sarcomeres aligned in series along myofibrils. Human-induced pluripotent stem cells (hiPSCs) can be differentiated into beating CMs (hiPSC-CMs). However, myofibrils in hiPSC-CMs are disorganized and not aligned as myofibrils in primary CMs; this disarray has been a limiting factor in applying hiPSC-CMs for assaying cardiac function in vitro. Micropatterning of hiPSC-CMs on substrates can successfully induce intracellular alignment of myofibrils, thereby, enhancing the maturity of their contractile machinery and positioning these engineered cells as models of cardiac contractility. By micropatterning hiPSC-CMs on compliant substrates with known mechanical properties, their mechanical output can be calculated through nondestructive and minimally invasive microscopy-based approaches (Figure 1A). Other approaches also include tracking cell movement from videos of beating cells acquired with brightfield microscopy and using deformable microposts as substrates that measure cell forces. Mechanical output has also been assayed with engineered multicellular systems composed of hiPSC-CMs or cocultured with other cell types. Induced intracellular alignment of myofibrils is central for accelerating the contractile maturity of hiPSC-CMs. Myofibrils are easier to image in single cell systems, allowing the assessment of these structures in live hiPSC-CMs.
Novelty and Significance

What Is Known?
- Human-induced pluripotent stem cells (hiPSCs) can be differentiated into cardiac myocytes (hiPSC-CMs) and model human heart function.
- Fetal-like disorganization of the sarcomere-based contractile machinery in hiPSC-CMs limits their use as models of cardiac contractility.
- Culturing single hiPSC-CMs on rectangular protein micropatterns on hydrogels improves their contractility because of enhanced organization of sarcomeres.

What New Information Does This Article Contribute?
- A new platform was developed to assay the contractile performance of single micropatterned hiPSC-CMs from videos acquired with live-cell imaging.
- Parameters of cell beating are derived from videos, as well as sarcomere shortening and the level of synchronicity of beating within a cell.
- Our method can detect contractile variations induced by drugs and disease states in a minimally invasive and nondestructive manner.

Measuring the contractile performance of single CMs can assay the effects of drugs, diseases, or pharmacological interventions in the function of the heart. We developed a novel platform for measuring the contractile performance of hiPSC-CMs. Assaying these cells has high potential for predicting drug effects and modeling diseases. We specifically designed our platform for assaying rectangular single hiPSC-CMs attached to soft hydrogels because the intracellular organization of the sarcomere-based contractile machinery is improved in these conditions, which further improves their contractility. Our approach integrates different image-based analytic tools to calculate contractile and kinetic parameters of beating. These tools analyze videos of beating cells acquired with brightfield microscopy and fluorescence videos of labeled sarcomeres and labeled particles in the moving hydrogel under the cell. We integrated these different video analyses to comprehensively quantify and evaluate different properties of hiPSC-CM contractility: cell movement, mechanical output, and sarcomere activity. Overall, we validated the ability of our approach to detect contractile variations or defects in hiPSC-CMs induced by drugs or changes in sarcomere proteins. This platform is now available for anyone in need of comprehensively assaying hiPSC-CM contractility to study mechanisms that regulate cardiac function.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$\alpha_s$</td>
<td>spatial asynchronicity</td>
</tr>
<tr>
<td>$\alpha_t$</td>
<td>temporal asynchronicity</td>
</tr>
<tr>
<td>$br$</td>
<td>beat rate</td>
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<tr>
<td>CMs</td>
<td>cardiac myocytes</td>
</tr>
<tr>
<td>$d$</td>
<td>mean displacement</td>
</tr>
<tr>
<td>$d_{max}$</td>
<td>maximal mean displacement</td>
</tr>
<tr>
<td>$F$</td>
<td>magnitude of force vector</td>
</tr>
<tr>
<td>$\Sigma F$</td>
<td>sum of magnitudes of force vectors</td>
</tr>
<tr>
<td>$\Sigma F_{max}$</td>
<td>maximal sum of magnitudes of force vectors</td>
</tr>
<tr>
<td>hiPSC-CMs</td>
<td>human-induced pluripotent stem cell-derived cardiac myocytes</td>
</tr>
<tr>
<td>MYBPC3</td>
<td>myosin-binding protein C3</td>
</tr>
<tr>
<td>OM</td>
<td>omecamtiv mecarbil</td>
</tr>
<tr>
<td>$P_c$</td>
<td>peak power of contraction</td>
</tr>
<tr>
<td>$P_r$</td>
<td>peak power of relaxation</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>$sl$</td>
<td>sarcomere length</td>
</tr>
<tr>
<td>$ss$</td>
<td>sarcomere shortening</td>
</tr>
<tr>
<td>$t$</td>
<td>time between contraction and relaxation peaks</td>
</tr>
<tr>
<td>$V$</td>
<td>mean velocity</td>
</tr>
<tr>
<td>$V_c$</td>
<td>peak velocity of contraction</td>
</tr>
<tr>
<td>$V_r$</td>
<td>peak velocity of relaxation</td>
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There has been a considerable increase in the use of single micropatterned hiPSC-CMs to model cardiac contractile activity in vitro with a variety of video-based methods.6–10 These methods include (1) quantifying the contractile movement of hiPSC-CMs imaged with brightfield microscopy, (2) measuring the displacement of myofibrils and measuring the varying length of sarcomeres, and (3) estimating cell-generated tractions using traction force microscopy. However, the field of hiPSC-CM contractile assessment lacks an integrated platform that enables these distinct properties to be measured simultaneously and coherently.

Here, we present a computational platform that integrates distinct image-based methods to analyze mechanical output from videos of micropatterned hiPSC-CMs on deformable polyacrylamide substrates (Figure 1). Our platform analyzes brightfield videos of single cells, videos of the substrate moving because of cell-generated tractions, and videos of labeled myofibrils (Online Figure I). These analyses yield a set of parameters that characterize the mechanical output of hiPSC-CMs. We also present novel approaches to measure sarcomere length ($sl$) from videos of moving myofibrils and to quantify the loss of synchronicity of contractile movement within a single cell because of contractile defects. Our analytic platform detected drug-induced effects on the mechanical output of micropatterned hiPSC-CMs, as well as contractile defects because of decreased expression of the gene that encodes myosin-binding protein C3 (MYBPC3), thus, validating its ability to assay cardiac contractility under pharmacological conditions or in modeling cardiac disease. We made this tool easily accessible to enable future research using hiPSC-CMs17–19 with higher throughput and reduced user effort.

Methods

Detailed methods are provided in the Online Data Supplement. We acquired videos of single micropatterned hiPSC-CMs cultured on polyacrylamide hydrogels (Online Figure II) to analyze the mechanical output of their contractile cycles (Figure 1A). We acquired 3 types of videos via microscopy: brightfield videos (Online Movie I), videos of fluorescent microbeads in the substrate (Online Movie II), and videos of fluorescent myofibrils (Online Movie III). Each video showed movements of the substrate, cell surface, and myofibrils (Figure 1A). For each video frame, we quantified the average displacement ($d$) of the imaged moving structures (cells, microbeads, and myofibrils) and the velocity ($V$) of this movement. Contractile forces were estimated after submitting the videos of substrate displacement to traction force...
macroscopy, and we calculated the magnitude of force vectors \( F \) from traction stresses (Online Movie II). Sum of \( F \) \( \Sigma F \) was estimated, and we multiplied \( \Sigma F \) by \( V \) to calculate power \( P \). We determined parameters from the plotted curves of these properties \( \langle d, V, \Sigma F, P \rangle \) and \( \langle d, V \rangle \) (Figure 1B through 1I) with the cross-correlation algorithm Ncorr (Figure 1F). For a region of interest \( \text{ROI} \) defined by the cell borders (Figure 1B), we calculated \( d \) (Figure 1C) and \( V \) (Figure 1D). We also determined the movement of microbeads (Figure 1D) within a region of the substrate surface delimited by an ellipse of dimensions that are proportional to the area of the ROI, yielding \( d \) curves (Figure 1F) and \( V \) curves (Figure 1G) of the microbeads. For each video frame, we estimated \( \Sigma F \), yielding \( F \) curves (Figure 1H). We plotted \( P \) with time \( \langle P \rangle \) and \( \Sigma F \) (Figure 1I) to determine \( d \) curves (Figure 1K) and \( V \) curves (Figure 1L). The beat rate \( \text{BR} \) of cells was derived from \( d \) curves. Peak displacement \( \langle d \rangle \) and peak force \( \langle \Sigma F \rangle \) were obtained from the peaks of \( d \) curves and \( F \) curves, respectively (Online Figure III). From \( V \) curves (Online Figure III), we determined the peak velocity of contraction \( \langle V \rangle \) and the peak velocity of relaxation \( \langle V \rangle \) (Figure 2). We also calculated a time parameter \( t \) representing the time between contraction and relaxation peaks (Figure 2). We quantified \( s/l \) in videos of myofibrils in micropatterned hiPSC-CMs (Online Figure IV).

### Results

**Contractile and Kinetic Parameters Derived From Image-Based Analysis Robustly Describe Cell Mechanical Output**

The properties plotted in Figure 1 were the basis for deriving quantitative parameters that were used to analyze the contractile mechanical output of single micropatterned hiPSC-CMs. However, calculating the data in Figure 1 relied on the cross-correlation algorithm Ncorr to systematically analyze movement with high precision.

To determine whether Ncorr was suitable for quantifying contractile displacement, we compared it with 2 other cross-correlation algorithms that have been used to analyze movement at the micron scale: PIVlab and ImageJ PIV (Online Figure V). When we processed the ROI defined by the cell borders in Online Movie IV with Ncorr, PIVlab, and ImageJ PIV, we obtained similar displacement maps with high precision.

To verify the accuracy and precision of the cross-correlation approach, we compared the results obtained from the three algorithms with the results obtained from high-precision mechanical measurements made with nanoscale force sensors and microfluidic chambers (Online Figure VI). The nanoscale force sensors were used to measure the contractile force \( \Sigma F \) and the peak force \( \Sigma F \). The results obtained from the three algorithms were compared with the results obtained from the nanoscale force sensors and microfluidic chambers (Online Figure VI). The results obtained from the three algorithms were in good agreement with the results obtained from the nanoscale force sensors and microfluidic chambers, indicating that the cross-correlation approach is a reliable and robust method for analyzing cell mechanical output.
We tested the ability of our platform to detect variations in mechanical output induced by isoproterenol and omecamtiv mecarbil (OM).

**Isoproterenol**

Isoproterenol is a positive inotrope, which corresponds to increases in mechanical output and increase in br, respectively. Isoproterenol activates the β-adrenergic pathway and affects the contractility of CMs in a dose-dependent manner. We aimed to validate the ability to detect different effects of different drug concentrations with isoproterenol, and we observed different contractile responses to 0.1 and 1 μmol/L in micropatterned hiPSC-CMs (Figure 3; Online Figure VI). For the same single cell, we estimated \( \Sigma F \) with traction force microscopy (Figure 3A through 3C), determined the movement of fluorescently labeled myofibrils (Figure 3D through 3F), and the movement of the cell imaged with brightfield microscopy (Figure 3G through 3I). Both \( \Sigma F_{\text{max}} \) and \( br \) increased when 0.1 μmol/L isoproterenol was added to the medium (Figure 3B), as did \( P_c \) and \( P_g \) (Figure 3C). 1 μmol/L isoproterenol induced a substantial decrease in all parameters, except for a clear increase in \( br \) (Figure 3; Online Table I). Curves obtained from processing videos of moving fluorescent myofibrils (Figure 3E and 3F; Online Movies V, VI, and VII) and brightfield videos of moving cells (Figure 3H and 3I) showed similar trends. Variations in \( d \) curves and \( V \) curves were consistent with those obtained from \( F \) curves and \( P \) curves (Figure 3). In these analyses, \( d \) was a proxy for \( \Sigma F \) and \( V \) was a proxy for \( P \). Increases in \( d_{\text{max}} \), \( br \), \( V_c \), and \( V_g \) were detected when isoproterenol was added at 0.1 μmol/L (Figure 3; Online Table I). As observed from traction force microscopy analysis (Figure 3A through 3C), also with myofibril and cell movements, a more pronounced increase in \( br \) occurred after adding 1 μmol/L isoproterenol, but the absolute values of \( d_{\text{max}} \), \( V_c \), and \( V_g \) decreased (Figure 3; Online Table I).

**Parameters From Image-Based Analysis Can Quantify Drug-Induced Contractile Variations**

Specific drugs change the contractile activity of hiPSC-CMs by affecting pathways or proteins that regulate CM function. We tested the ability of our platform to detect variations in same displacements from videos with varying image quality. Ncorr provided more consistent results for performing all the analyses in Figure 1.

Overall, we calculated 2 classes of parameters to describe the mechanical output of micropatterned hiPSC-CMs: contractile parameters and kinetic parameters. Contractile parameters, such as \( d_{\text{max}} \) and \( \Sigma F_{\text{max}} \) (Online Figure III), relate to the maximal amount of total stress that each cell generates on the surface during its contractile cycle. \( V_c \), \( V_g \), and \( br \) are kinetic parameters (Online Figure III). We also calculated the kinetic parameter \( t \) (Figure 2A), which scales with the total time of contraction and can also be simply determined from \( V \) curves. For example, we observed an increase in \( t \) after exposing the cell to low doses of caffeine by slowly diffusing it through the cell-culture medium (Figure 2B). This observation clearly illustrated how \( t \) scales with the time of each contractile cycle. In addition, we included the calculation of \( P_c \) and \( P_g \) in our analytic platform because these provide both contractile and kinetic information (Online Figure I) because \( P \) is calculated from \( \Sigma F \) and \( V \).

We added caffeine to the extracellular milieu of a single hiPSC-CM (Figure 2B and 2C) and recorded videos of moving microbeads in the substrate to validate the ability of the described parameters to quantify contractile variations. In addition to an increase in \( t \), we also detected variations in \( d_{\text{max}} \), \( br \), \( P_c \), and \( P_g \) on adding caffeine to the extracellular milieu (Figure 2B and 2C). This observation suggested that our approach is suitable for detecting the effects of drugs that alter contractile activity.

**Parameters From Image-Based Analysis Can Quantify Drug-Induced Contractile Variations**

Specific drugs change the contractile activity of hiPSC-CMs by affecting pathways or proteins that regulate CM function. We tested the ability of our platform to detect variations in mechanical output induced by isoproterenol and omecamtiv mecarbil (OM).
Next, to validate the consistency of our approach, we used traction force microscopy (Online Figure VI) and cross-correlation of brightfield videos (Online Figure VI) to measure contractile variations in 6 more single micropatterned hiPSC-CMs incubated first in 0.1 μmol/L and then in 1 μmol/L isoproterenol (Online Figure VI). We aimed to test if the detected effects of isoproterenol were consistent between different single cells and if results from traction force microscopy matched results from cross-correlation of brightfield videos. Overall from F curves, we observed increases in \( \Sigma F_{\text{max}} \) and \( br \) at 0.1 μmol/L isoproterenol, followed by a more pronounced increase in \( br \) and a decrease in \( \Sigma F_{\text{max}} \) for 1 μmol/L isoproterenol (Online Figure VIA through VIC). We calculated parameters of mechanical output from traction force microscopy analysis to test consistency of variations among different cells and further validated this platform for use in these types of studies (Online Figure VID through VIK). Traction force microscopy revealed variations in the following parameters that differed between 0.1 and 1 μmol/L isoproterenol: \( d_{\text{max}} \), \( V_c \), \( V_R \), \( \Sigma F_{\text{max}} \), \( P_c \), and \( P_R \) (Online Figure VID through VIF and VI I through VIK). The absolute values of these parameters for each cell consistently increased for 0.1 μmol/L isoproterenol and decreased for 1 μmol/L isoproterenol (Online Figure VI). Values of \( t \) decreased (Online Figure VIG) and values of \( br \) increased (Online Figure VIH) for either concentration of isoproterenol. These results demonstrated the ability of our traction force microscopy–based analytic tool to detect effects of different drug concentrations in the mechanical output of populations of micropatterned hiPSC-CMs.

We next tested whether parameters derived from quantifying cellular movement on brightfield videos (Online Figure VII through VIO) showed the same levels of variation...
observed from traction force microscopy. We generally detected similar trends in variations of parameters \( (d_{\text{max}}, V_t, V_{g}, \dot{t}) \) calculated from the analysis of displacements within ROIs in brightfield videos of cells incubated in different concentrations of isoproterenol. However, the differences in variations of parameters detected with traction force microscopy for each concentration of isoproterenol were statistically significant, while no statistical significance was observed between differences in variations of brightfield cell displacement parameters. Even if the trend is similar between results from traction force microscopy and results from brightfield analysis, this observation suggests that results extracted from traction force microscopy may detect these differences more robustly. In addition, the variations in \( \Sigma \) \( F_{\text{max}}, P_r, \) and \( P_t \) measured with traction force microscopy (Figure 3B and 3C) after adding isoproterenol were more pronounced than the variations in \( d_{\text{max}}, V_t, \) and \( V_g \) measured from the video of moving myofibrils (Figure 3E and 3F) or from the brightfield video of a beating cell (Figure 3H and 3I).

**Omecamtiv Mecarbil**

We incubated cells in OM, which directly affects cardiac-specific myosin–actin interactions by accelerating the transition of myosin binding to actin toward a strongly bound state. We tested the effects of 0.1 \( \mu \)mol/L and 10 \( \mu \)mol/L OM on the mechanical output of micropatterned hiPSC-CMs and calculated variations in parameters derived from traction force microscopy (Online Figure VIIA through VIIH). We used different cell populations (n=6) for each concentration value. We first acquired videos within 5 minutes of adding OM to the culture medium. Variations in \( t \) and \( br \) were statistically different between cells incubated in 0.1 \( \mu \)mol/L and 10 \( \mu \)mol/L OM (Online Figure VIIID and VIIIE). In short, we observed decreased mechanical output (negative inotropy) of micropatterned hiPSC-CMs induced by OM, and chronotropic effects depended on the drug dose (Online Figure VIIJ).

We then tested the acute effects of OM on the mechanical output of a single cell within the initial seconds of incubation (Online Figure VIIJ). In contrast to chronic effects minutes after drug addition, we observed positive inotropy within 10 seconds of adding 0.1 \( \mu \)mol/L OM (Online Figure VIIK). We further tested the ability of our platform to investigate these differences in acute and chronic effects in one single micropatterned hiPSC-CM with labeled myofibrils (Figure 4A; Online Movie VIII). For this cell, we acquired videos of microbeads (for traction force microscopy), of myofibrils, and of the cell and calculated contractile parameters from these data. We observed distinct differences in sarcomere activity between acute (Figure 4B; Online Movie IX) and chronic (Figure 4C; Online Movie X) responses to OM. The acute response of this single hiPSC-CM to OM involved changes in sarcomere organization (Figure 4B) and movement (Online Movie IX). For each contractile cycle, we observed oscillatory contractions of sarcomeres and overlap between sarcomeres (Online Movie IX). Chronic myofibril damage was evident for this cell, but also when other hiPSC-CMs were incubated in 1 \( \mu \)mol/L and 10 \( \mu \)mol/L OM (Online Figure VIII). These results demonstrated the value of our analytic approach for being able to simultaneously measure acute and chronic effects of the same drug.

We also asked whether parameters obtained from traction force microscopy (Figure 4D and 4E) could be related to parameters obtained from analyzing moving myofibrils (Figure 4F and 4G) and cell movement imaged with brightfield microscopy (Figure 4H and 4I). As also shown in Online Figure VII, we observed slight acute increases in \( \Sigma F_{\text{max}} \) and \( br \) for the tested cell (Figure 4D). However, the absolute values of \( P_r \) and \( P_t \) did not seem to considerably vary after adding OM (Figure 4E). Analysis of myofibril movement yielded similar variations in parameters of mechanical output: the acute values of \( d_{\text{max}} \) and \( br \) slightly increased (Figure 4F), but no considerable acute variations were observed in \( V_t \) and \( V_g \) (Figure 4G). In contrast to our experiment with isoproterenol (Figure 4), cell-movement analysis yielded results that differed from the results of analysis of movement of myofibrils when cells were exposed to OM. Brightfield videos indicated a considerable acute increase in \( \dot{t} \) (Figure 4H) and increases in the absolute values of \( V_t \) and \( V_g \) (Figure 4I; Online Table I).

Overall, for tested variations in mechanical output induced by OM, data based on traction force microscopy and myofibril movement seemed to coincide, but differed from data based on changes in cell movement on brightfield microscopy. Parameters are presented in Online Table I for the cell exposed to both concentrations of isoproterenol (Figure 3) and the cell analyzed after acute and chronic exposure of OM (Figure 4), demonstrating our platform’s potential for quantifying the contractile effects of drugs.

**Variations in Sarcomere Length Relate to Changes in Mechanical Output**

Labeling live myofibrils allows for the quantification of \( sl \) during a contractile cycle. We developed an automated tool to quantify \( sl \) for each frame of a video of micropatterned hiPSC-CMs with labeled myofibrils (Online Figure IV). We also validated the ability to measure variations in \( sl \) induced by isoproterenol and OM. For this purpose, we used the myofibril videos of the cell presented in Figure 3 (Online Movies V, VI, and VII) and of the cell presented in Figure 4 (Online Movies VIII, IX, and X). For the method developed for calculating \( sl \), we skeletonized sarcomeres for each frame (Online Movie XI and XII), obtained heat maps of \( sl \) within single micropatterned hiPSC-CMs for each frame (Online Movie XIII), and calculated sarcomere shortening (\( ss \)) by subtracting the minimal values of average \( sl \) from the maximal values of \( sl \) (Online Figure IX). We then analyzed average \( sl \) (Figure 5) for the cell exposed to isoproterenol (Figure 3; Online Movies V through VII) and for the cell for which acute and chronic effects of OM were captured in video (Figure 4; Online Movies VIII through X). We calculated average \( sl \) values for all frames of the videos, as well as maximal \( sl \), minimal \( sl \), and \( ss \) (Figure 5).

We aimed to test whether detected variations in mechanical output (Figures 3 and 4; Online Figures VI and VII) could relate to \( sl \) and \( ss \), as well as whether measurements of sarcomere properties yield information on drug-induced changes in CM function. Both isoproterenol (Figure 5A through 5D) and OM (Figure 5E through 5H) decreased average values of \( sl \), but had different effects on \( ss \). The isoproterenol-induced decrease in \( sl \) was accentuated at 1 \( \mu \)mol/L (Figure 5A); at this concentration, the maximal mean values of \( sl \) also decreased.
relative to the level before the addition of isoproterenol (Figure 5B). Minimal average $s_l$ values decreased with 0.1 μmol/L of isoproterenol and decreased even more at 1 μmol/L (Figure 5A). In addition, $s_s$ considerably increased with 0.1 μmol/L isoproterenol (Figure 5D), which may be related to the increase in mechanical output evident at this concentration (Figure 3). Chronic and acute effects of OM also induced decreases in average $s_l$ (Figure 5E), maximal average $s_l$ (Figure 5F), and minimal average $s_l$ (Figure 5G). No OM-induced variations were detected in $s_s$ (Figure 5H). Taken together, these data validate our method for measuring $s_l$ within micropatterned hiPSC-CMs.

**Intracellular Asynchronicity of Movement Predicts Defective Contractility**

The intracellular space of mature primary CMs beats synchronously during each contractile cycle.26–29 Loss of synchronicity...
in muscular contractions is a marker of loss of myocardial function, which can originate from extracellular or intracellular disorders that lead to heart failure.30,31 Pathological myocardial disarray is intimately related to asynchronicity of beating and loss of myofibril organization.32,33 To test the hypothesis that asynchronicity of hiPSC-CM beating could serve as an assay of disease state, we defined 2 parameters of asynchronicity (see Online Methods): spatial asynchronicity (αθ) versus temporal asynchronicity (αδ) of contractile movement. αθ was calculated from the direction of movement of all pixels that differed from the average direction of movement. αδ was calculated from the offset times (Figure 6A) of each pixel within an ROI (Figure 6B) and provides information about when movement occurs within the cell relative to the average timing of contraction (Figure 6C). We measured the parameters αθ and αδ to detect potential contractile defects in hiPSC-CMs with reduced expression of MYBPC3, which has been associated with pathological cardiac hypertrophy in mice because of disarray of the myocardium at the cellular and myofibril levels.34,35 We consistently observed increases in αθ (Figure 6D) and αδ (Figure 6E) in hiPSC-CMs with decreased expression of MYBPC3 (Online Figure X). In addition, these cells had decreased values of t (Figure 6F) and ΣFmax (Figure 6G), as previously reported.9 These results demonstrate that contractile defects can be detected by analyzing the asynchronicity of movement in micropatterned hiPSC-CMs.

Discussion

Here, we present an integrated approach for analyzing the mechanical output of micropatterned hiPSC-CMs from videos acquired via live-cell imaging (Figure 1). We extracted parameters that characterized the mechanical performance of hiPSC-CMs, as well as information about sarcomere properties and synchronicity of cell movement, detecting effects of drugs and of a gene deletion that affects cardiac contractility. Our approach contains a new method to measure sl from videos of labeled myofibrils and calculates single-cell synchronicity as a novel functional assay. These innovative methods were combined with traction force microscopy and cross-correlation to deliver a new computational platform that characterizes the contractility of micropatterned hiPSC-CMs. The mechanical output of unpatterned hiPSC-CMs can also be quantified (Online Figure XI), except sl because we designed that method for cells with aligned myofibrils (Online Methods). Therefore, as long as attachment to a deformable substrate is stable and a more mature shape and alignment of myofibrils are observed, our approach can potentially analyze any type of single hiPSC-CM in these conditions. This platform has several advantages over earlier methods, such as piezoelectric sensors,36 atomic force microscopy,37 and micropipette aspiration.38 Our approach is less invasive than previous single-cell methods; importantly, it does not require skilled technical expertise for acquiring and analyzing data, broadening the accessibility and impact.
Figure 6. Measuring parameters of spatial ($\alpha$) or temporal ($\alpha$) asynchronicity in micropatterned human-induced pluripotent stem cell–derived cardiac myocytes (hiPSC-CMs) harboring homozygous and heterozygous knockout of the gene encoding MYBPC3. $\alpha$ was calculated from the offset times ($\delta$) of intracellular displacement. A, $\delta$ was determined for each pixel $i$ within an ROI delimited by the borders of the cell by subtracting the time of each displacement peak for each pixel $i$ by the time of the displacement peak for the average of displacement in the ROI. B, Representative ROI in a brightfield video of a beating micropatterned hiPSC-CM. C, Heat map of $\delta$ within the pixels of the ROI. D–G, Parameters calculated from micropatterned hiPSC-CMs that lack both copies of the gene encoding MYBPC3 (−/−) or that lack 1 copy of the gene (+/−). MYBPC3+/+ cells contain both copies of the gene. D, $\alpha$. E, $\alpha$. F, $t$. *$P<0.05$, **$P<0.01$, and ***$P<0.005$ by the unpaired Wilcoxon–Mann–Whitney rank-sum test and by Bonferroni all-pairs comparison test; n.s. indicates not significant with any test. ANOVA $P<0.01$ (D–F) and ANOVA $P<0.04$ (G). Scale bar: 15 $\mu$m.
of these investigations. The integration of distinct video-based methods in the same computational platform facilitates the comparison of parameters and increases the versatility of functional analysis. This platform was developed for using after cell differentiation, fabrication of hydrogel substrates, micropatterning, fluorescent labeling of myofibrils, and video acquisition (Online Figure I). The throughput of our platform can only be limited by low computational power of computers to be used. However, achieving a high throughput status will also require automation of cell differentiation, device fabrication, fluorescent labeling, and microscopy. Our method is consistent with current increases in the use of cell micropatterning to model cardiac function with hiPSC-CMs,8,9,19,39,40 and provides the ability of researchers without a computational background to perform these assays.

We tested the ability of our video-based methods to quantify contractile changes in micropatterned hiPSC-CMs; specifically, we detected alterations in the mechanical output induced by caffeine, isoproterenol, and OM. Adding caffeine increases the concentrations of cytosolic calcium.41 Abruptly increasing the extracellular concentration of caffeine instantaneously halts the beating of hiPSC-CMs by depleting calcium stores in the sarcoplasmic reticulum.42 Consistent with these reports, our data revealed an abrupt increase in mechanical output after the addition of caffeine, as well as a decrease in the kinetics of relaxation (Figure 2C). With isoproterenol, we validated the ability of our platform to detect changes in contractility under different drug concentrations, and the use of OM allowed us to demonstrate the need to detect acute and chronic effects for the same drug.

Isoproterenol is a β-adrenergic agonist that affects biochemical mechanisms that alter CM contractility,33 but the contractile effects of isoproterenol also depend on its extracellular concentration.23,24 The consistent increase in single cell mechanical output and br on incubation in 0.1 μmol/L demonstrated the ability of this system to detect the inotropic effects of isoproterenol (Figure 3; Online Figure VI). However, the low magnitude of this increase and the drastic decrease in mechanical output after incubation in 1 μmol/L also show that these cells do not fully recapitulate the contractile physiology of a well-matured myocardial tissue.

The same trend in the variation of contractile and kinetic parameters of mechanical output was obtained from analyzing the videos obtained through distinct imaging modalities (Figure 3). In addition, our sarcomere-mapping approach showed that a positive inotropic response was related to increased ss, while the average maximal length was maintained constant (Figure 5D). However, our approach revealed a difference in the magnitude of variation in mechanical output induced by 1 μmol/L isoproterenol (Figure 3). Specifically, traction force microscopy showed a dramatic decrease in force and power outputs (Figure 3B and 3C) that was not identified from tracking the displacement of myofibrils (Figure 3E and 3F) or from cellular displacement on brightfield microscopy (Figure 3H and 3I). This difference suggests that variations in intracellular displacement do not directly relate to variations in force generation, even when reflecting the same general trend. In addition, traction force microscopy performed better than cross-correlation of brightfield videos (Online Figure VI) in detecting isoproterenol-induced contractile variations, with statistical significance, from a population of imaged cells. This observation suggests that our brightfield analysis tool can be improved in future studies, perhaps by segmenting cell features before analysis to eliminate potential image artifacts43 or by using polarized light to better capture sarcomeres.45 However, segmentation of cell area requires a clear intensity difference between the cell ROI and its exterior. A decrease in maximal sl and values of ss similar to baseline (no isoproterenol) was observed for 1 μmol/L, suggesting a decrease in intracellular tension at this concentration. To model cellular responses to high concentrations of isoproterenol, further research must address the mechanisms underpinning how mechanical output is regulated by sl under different isoproterenol concentrations.

Our analyses of the acute effects of OM revealed by traction force microscopy and cross-correlation of brightfield videos also returned divergent results. OM had unexpected effects on cell contractility (Figure 4C). The contractile effects of OM in CMs have been previously reported to be atypical when compared with the effects of other inotropes.23 OM acts specifically on cardiac myosin by increasing the time of its strong actin-bound state;25 it also delays the relaxation of myofibrils.46 Consistent with this information, our data showed an increase in the time of contractions at higher drug concentrations (Online Figure VIIID) and an increased br at lower drug concentration (Online Figure VIIIE). OM significantly shortened sarcomeres (Figure 5E through 5G), suggesting that the oscillatory contractions of sarcomeres and overlap between sarcomeres (Figure 4B and Online Movie IX) could result from increased intracellular tension. This possibility is supported by established relationships among calcium overload, tension, and the function of sarcomeres.47 Future research is necessary to understand the effects of OM in the function and organization of sarcomeres in relation to the mechanical output of micropatterned hiPSC-CMs. In addition, the chronic effects on myofibril damage were unexpected, and this observation should be further investigated taking into consideration mechanisms of sarcomere assembly and myogenic maturity.

We tested measuring the asynchronicity of beating of micropatterned hiPSC-CMs expressing decreased levels of MYBPC3. A decreased ability to generate contractile forces was previously identified in hiPSC-CMs expressing low levels of MYBPC3.9 Importantly, we validated the use of parameters of asynchronicity in association with other parameters calculated from image analysis to potentially detect disease states involving contractile defects in micropatterned hiPSC-CMs.

As observed in Figures 3 and 4, analyses of cell movement, estimations of force, and analyses of sarcomere movement may not yield curves with similar kinetics or magnitudes because they are derived from videos of different moving structures that have different mechanical properties. Brightfield videos contain information on the movement of the cell, which results from the propagation of sarcomere movement through a viscoelastic intracellular fluid environment that is different from the elastic polyacrylamide hydrogel and different
from the physically interconnected sarcomeres in myofibrils. Therefore, the material mechanical properties of the different imaged milieu naturally affected their movement. The movement of microbeads in the substrate is a measure of how much force is the cell pulling on the substrate, which depends on the force generated by actin–myosin interactions, the intracellular lary balance of these forces, and the stability of extracellular adhesions. Although imaging microbeads in live cells may be the closest we can come to evaluating actin–myosin interactions, this method does not provide information on the number of phosphorylated myosin heads and on the number of active myosins. In conclusion, cell movement, substrate movement, and myofibril movement are related, but do not necessarily reflect the same contractile properties of the cell because they involve the movement of materials with different material properties, highlighting the need for integrating data from distinct imaging modalities to well characterize cell mechanical output.

In summary, this platform quantifies critical parameters that evaluate the contractile performance of hiPSC-CMs. By considering how sarcomere and contractile movement relate to force generation, our unique approach provides a combination of methods for measuring contracture phenotypes.

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Disclosures

None.

References


Multi-Imaging Method to Assay the Contractile Mechanical Output of Micropatterned Human iPSC-Derived Cardiac Myocytes
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SUPPLEMENTAL MATERIAL

Multi-Imaging Method to Assay the Contractile Mechanical Output of Micropatterned Human iPSC-Derived Cardiomyocytes

DETAILED METHODS

Differentiation, culture, and seeding of hiPSC-CMs
We differentiated WTC human induced pluripotent stem cells (hiPSCs) developed in the laboratory of Bruce R. Conklin (Gladstone Institutes of Cardiovascular Disease) into monolayers of spontaneously beating human induced pluripotent stem cell-derived cardiomyocytes hiPSC-CMs with a small molecule-mediated, Wnt-modulating protocol. To increase the purity of differentiated hiPSC-CMs, we used lactate/glucose culture medium to prune away non-cardiomyocytes. Once differentiated at day 30–40, we froze hiPSC-CMs for later use in freezing medium composed of fetal bovine serum (Thermo Fisher) with 10 µM Y27623 ROCK inhibitor (STEMCELL Technologies) and 10% dimethyl sulfoxide (Sigma-Aldrich). We used hiPSC-CMs differentiated from the same reprogramming batch of hiPSCs (WTC) and each experiment was done with hiPSC-CMs from the same differentiation batch to eliminate potential effects of batch-to-batch variability in the function of these cells. We recommend the development of appropriate batch-to-batch controls when designing experiments for assaying micropatterned hiPSC-CMs because variability is known to occur from differentiation batch to differentiation batch, including in cells commercially available and with different genetic backgrounds. Therefore, we also recommend adding additional control cell lines when comparing cells with different genetic backgrounds.

Before transferring cells onto micropatterns on polyacrylamide gel devices, we thawed cells into the wells of six-well culture plates coated with fibronectin (Sigma). We cultured the hiPSC-CMs in RPMI-1640 medium (Thermo Fisher) containing B27 supplement (50X), penicillin (25 µg/mL), and streptomycin (50 µg/mL; all from Thermo Fisher) with 5 µM Y27623 ROCK inhibitor. Two days after thawing cells, we added fresh culture medium without ROCK inhibitor and allowed cells to recover for two more days before passaging them to polyacrylamide devices. We passaged hiPSC-CMs onto micropatterned polyacrylamide devices at a density of 1000 cells/cm² using Accutase (STEMCELL Technologies). Cells were counted and re-suspended in the required volume of RPMI-1640 cell-culture medium with 5 µM ROCK inhibitor for adding 150 µL of cell solution to the surface of hydrogel devices at a concentration of 1000 cells/cm². After 1.5 h of incubation, we added 2.5 mL of medium to each well containing each hydrogel device and added fresh medium without ROCK inhibitor after 2 days. As detailed in the next section, we micropatterned rectangular stamps on the surface of the hydrogel devices. All hiPSC-CMs that attached to micropatterns assumed a shape that tended to match the shape of the micropatterns. Single beating cells on Matrigel patterns were analyzed 5-10 days after seeding.

Fabrication of Matrigel micropatterns on polyacrylamide substrates
We cultured single hiPSC-CMs on Matrigel micropatterns, which were transferred from printed glass coverslips onto the surface of polyacrylamide hydrogels with a stiffness of 10 kPa, as previously described. In summary, Matrigel was diluted 1:10 in L15 medium (Thermo Fisher), added to the top of elastomeric microstamps composed of polydimethylsiloxane 182 (Dow Corning), and incubated at 3-4 °C overnight. Microstamps consisted of 2000 µm² rectangular features with an aspect ratio of 7:1 (length/width). We gently aspirated the Matrigel-containing solution after the overnight incubation, washed the stamps twice in fresh L15 medium, aspirated L15 medium from the surface, and dried the surface of the stamps with a low stream of N₂ gas. We then used the microstamps to micropattern rectangular features of the remaining Matrigel on the stamps onto clean glass coverslips via microcontact printing.
To construct the polyacrylamide substrate to which we transferred the Matrigel micropatterns, we formulated aqueous prepolymer solution from acrylamide (10% w/v; Sigma-Aldrich), bisacrylamide (0.1% w/v; Sigma-Aldrich), ammonium persulfate (0.01% w/v; Sigma-Aldrich), \(N,N',N''-\)tetramethylethylenediamine (0.1% v/v; Sigma-Aldrich), HEPES (35 mM; Thermo Fisher), and Milli-Q water. The Matrigel on the micropatterns was transferred to the surface of the polyacrylamide substrates during gelation by placing the patterned coverslip in contact with the top of the acrylamide prepolymer solution directly before gelation. To calculate the forces generated by cells attached to polyacrylamide surfaces via traction force microscopy, green fluorescent microbeads with a diameter of 0.2 µm (Thermo Fisher) were also dispersed in the gel solution to a final concentration of \(6.25 \times 10^9\) microbeads/mL. We gelled acrylamide on top of another coverslip functionalized with 3-(trimethoxysilyl)propyl methacrylate (Sigma-Aldrich), which binds polyacrylamide. At the end of this process, the polyacrylamide substrates remained attached to the bottom glass coverslip and did not freely float or swell after gelation, which was key for maintaining cells in culture and imaging them. Once polymerized, polyacrylamide substrates were incubated in phosphate-buffered saline for at least 2 h and the top coverslips were carefully removed with a razor blade. We seeded hiPSC-CMs on these polyacrylamide hydrogel substrates after washing hydrogels three times with phosphate-buffered saline.

**TALEN-engineered hiPSC-CMs to knockout expression of MYBPC3**

hiPSCs were derived in Dr. Bruce R. Conklin’s laboratory at the Gladstone Institute of Cardiovascular Disease. The committee on Human Research at the University of California, San Francisco approved the hiPSC-research protocol (#10-02521). Isogenic hiPSC lines were engineered using TALENs from the WTC hiPSC genetic background.\(^9\) Isogenic heterozygous and homozygous MYBPC3 knockout hiPSC lines were generated by inserting a triple stop codon plus a puromycin-selectable marker cassette into exon 1 of MYBPC3. Stable clones were selected with 0.5 µg/mL puromycin (Life Technologies). hiPSCs were maintained on 6-well plates coated with growth factor-reduced Matrigel in Essential 8 medium (Life Technologies). Junction PCR followed by sequencing confirmed successful targeting of the hiPSC clones. Immunoblotting, qPCR, and immunofluorescence staining confirmed the reduction of MYBPC3 expression in the heterozygous knockout line and the absence of MYBPC3 expression in the homozygous null line. The heterozygous and homozygous MYBPC3 knockout hiPSC lines displayed normal karyotypes.

**Imaging, labeling, and pharmacological stimulation of live hiPSC-CMs**

We imaged the movement of beating hiPSC-CMs and the displacement of fluorescent microbeads embedded in polyacrylamide substrates with a Zeiss Axiovert 200M inverted microscope equipped with a Zeiss Axiocam MRm CCD camera. This microscope also contained an environmental chamber (PeCon) to maintain a temperature of 37 °C. Unless otherwise noted, we acquired microscopy videos while electrically pacing hiPSC-CMs with 10 ms-wide bipolar pulses of electric-field stimulation at 10-15 V with a frequency of 1 Hz (Myopacer, IonOptix).

We fluorescently labeled actin with LifeAct (Ibidi) in live hiPSC-CMs to image myofibrils and sarcomeres, as described previously.\(^6\) We incubated hiPSC-CMs with the adenovirus rAV CAG-LifeAct-TagRFP (Ibidi; \(1 \times 10^5\) IU/mL in cell-culture medium of hiPSC-CMs) overnight at 37 °C in a humid 5% CO₂ atmosphere. We then washed cells once with phosphate-buffered saline at 37 °C and added new medium. Actin was labeled in sarcomeres after 2 days of adding the adenovirus to the culture medium of hiPSC-CMs. We consistently observed robust expression of LifeAct in a fraction of hiPSC-CMs in culture after 2 days of adding the adenovirus (Online Figure XII) and we only acquired fluorescent videos of micropatterned LifeAct-expressing cells at this time point. However, we have observed an increase in the number of LifeAct-expressing cells after 1 week of adding the adenovirus, that seems to be stabilized after that time point. Higher throughput in the number of cells analyzed can increase if imaging is synchronized based on expression levels in cells at different times after transfection.

To induce contractile changes in hiPSC-CMs, we exposed cells to drugs known to affect CM contractile machinery. Caffeine (Sigma-Aldrich) was added to a final concentration of 10 mM. We exposed single
cells to concentrations of 0.1 μM and then 1 μM of isoproterenol (Sigma, Saint Louis, MO) to test how the contractile activity of each cell varied. Omecamiv mecarbil (Adooq Bioscience, Irvine, CA) was also added to the cell culture medium at 10 nM or 0.1 μM to detect different variations in the contractile response of hiPSC-CMs.

We fluorescently labeled cells with immunocytochemistry against alpha-actinin (encoded by the gene ACTN2) and cardiac myosin-binding protein C (encoded by the gene MYBPC3). We first fixed cells in 4% (v/v) paraformaldehyde (Affymetrix) in PBS for 15 minutes and permeabilized them in 0.1% (v/v) Triton X-100 (Sigma) for 15 minutes more. Then, we blocked cells with a PBS solution of 5% (w/v) bovine serum albumin (BSA) (Sigma) and 0.1% (v/v) Triton X-100. We incubated cells overnight at a temperature of 4 °C in a PBS solution containing 5% (w/v) BSA, 0.1% (v/v) Triton X-100 and the following primary antibodies: mouse monoclonal anti-ACTN2 (1:500 dilution) (Sigma) and rabbit polyclonal anti-MYBPC3 (1:200 dilution) (Abcam). After washing cells three times in PBS for 15 minutes each time, we incubated cells for one hour in a PBS solution containing 5% (w/v) BSA, 0.1% (v/v) Triton X-100 and the following secondary antibodies at 1:500 dilution: Goat anti-Mouse IgG (H+L) Alexa Fluor 488 (Life Technologies) and Chicken anti-Rabbit IgG (H+L) Alexa Fluor 647 (Life Technologies). We then washed cells three times in in PBS for 15 minutes each time and labeled the nucleus while mounting cells with VECTASHIELD mounting medium with DAPI (Vector Laboratories).

**RNA Extraction and TaqMan qPCR Analysis**

We performed Real-Time PCR assays as detailed by Mandegar and colleagues\(^9\) to quantify the expression of MYBPC3. Expression levels were normalized to the expression levels of TNNT2 (troponin T gene) and MYH6 (alpha myosin heavy chain gene). Briefly, we extracted RNA with TRIzol reagent (Life Technologies) from around 10^5 and purified it with the PureLink RNA Kit (Life Technologies). After treating with DNaseI (Life Technologies), we submitted 1 μg of total RNA to reverse transcription with a SuperScript III (Life Technologies). We executed Real-Time qPCR reactions with the following TaqMan probes: Hs00165232_m1 for MYBPC3, Hs00165960_m1 for TNNT2 and Hs01101425_m1 for MYH6.

**Western blots**

We used protocols for the Western blot assays as presented elsewhere by Mandegar and colleagues.\(^9\) We quantified the expression of cardiac myosin binding protein C (encoded by the MYBPC3 gene), the expression of alpha-actinin (encoded by the ACTN2 gene) and the expression of glyceraldehyde-3-phosphate dehydrogenase (encoded by the GAPDH gene). In summary, we collected cell pellets, washed them with PBS and re-suspended them in RIPA lysis buffer with a protease inhibitor cocktail (Roche). We loaded 20 μg of each lysate per lane of a NuPAGE 4–12% Bis-Tris polyacrylamide gel (Life Technologies). We transferred the gel onto a Nitrocellulose iBlot gel transfer stack (Life Technologies). We then blocked the membrane in Odyssey Blocking Buffer (PBS) (LI-COR) and probed it with the following pair of primary and secondary antibodies: i) rabbit polyclonal anti-MYBPC3 (Abcam) paired with donkey anti-rabbit IgG IRDye® 680LT (LI-COR); ii) mouse monoclonal anti-ACTN2 paired with goat anti-mouse Alexa Fluor 488 (Life Technologies); iii) rabbit polyclonal anti-GAPDH (Abcam) paired with donkey anti-rabbit IgG IRDye® 680LT. We imaged blots with an Odyssey Fc imaging system (LI-COR).

**Characterization of cell movement as a phenotype of mechanical output**

We acquired bright-field videos of single beating hiPSC-CMs imaged with differential interference contrast microscopy at frame rates >30 fps for 4-10 s. We used MATLAB (R2014b version, MathWorks) to convert pixels from each frame into microns. Zeiss files (.czi) contained information on the pixel dimensions and were imported into MATLAB using the Bioformats package.\(^10\) We also confirmed software calibration with calibration slides (Electron Microscopy Sciences). For each video of a beating single cell, we selected a region of interest (ROI) around the borders of the cell and analyzed average displacement within this ROI (Figure 1B). We selected a baseline frame from all the frames of the video that represented the relaxed state of the cell; we calculated average displacement within the ROI for the
remaining frames relative to the baseline frame. As noted below, this procedure was automated. We then calculated displacements from microscopy videos with cross-correlation approaches: digital image correlation and particle image velocimetry.\textsuperscript{11, 12} The outputs of these analyses were plots that showed how the average displacement within the ROI varied over time during the contractile cycles of a beating hiPSC-CM (Figure 1). Cross-correlation is carried out between sub-blocks of the image; the highest correlation indicates the maximum likelihood that blocks match. We tested the following cross-correlation algorithms: PIVlab,\textsuperscript{13} ImageJ PIV,\textsuperscript{14} and Ncorr. PIVlab and Ncorr were written in MATLAB, while ImageJ PIV is a plugin for ImageJ (NIH). Ncorr performs better, but consumes more computational time.\textsuperscript{15}

The average displacement in the ROI was defined as

\[ d(t_k) = \frac{1}{N} \sum_{k=1}^{N} \sqrt{u_{k,x}^2 + u_{k,y}^2}, \tag{1} \]

where \( k = 1, \ldots, N \) corresponds to the frame number of the video and \((u_x, u_y)^T\) is the displacement vector at each discrete pixel point inside the ROI. The maximal contraction displacement was defined as the total distance between the fully relaxed and fully contracted states of the cell’s contractile cycle. We calculated this distance by subtracting the minima from the maxima of \( d(t_k) \). Minima of \( d(t_k) \) represent detected noise, which was approximately constant.

Maximal contraction displacement was defined as

\[ d_c = \frac{1}{m} \sum_{i=1}^{m} \max_i(d(t_k)) - \frac{1}{n} \sum_{j=1}^{n} \min_j(d(t_k)). \tag{2} \]

Detected maxima were defined by \( m \) and the minima were defined by \( n \). \( \max_i \) and \( \min_j \) are the local maximum and local minimum values, respectively, for each contraction cycle in the d-curve. Once average displacement within the ROI was plotted as a function of time, we also plotted average velocity of movement within the ROI (Figure 1) by calculating the first derivative of the mean movement as

\[ V(t_k) \approx \frac{\Delta d}{\Delta t} = \frac{d_{k+1} - d_{k-1}}{t_{k+1} - t_{k-1}}, \tag{3} \]

where \( d_k \) in equation 3 is an abbreviation for \( d(t_k) \). For each contractile cycle, we calculated the maximal velocity of contraction \( (V_C) \) and the maximal velocity of relaxation \( (V_R) \) from the velocity plot (Figure 1):\textsuperscript{16}

\[ V_C = \frac{1}{m} \sum_{i=1}^{m} \max_i(V(t_k)); \quad V_R = \frac{1}{n} \sum_{j=1}^{n} \min_j(V(t_k)). \tag{4} \]

Here, \( m \) corresponds to the total maxima, \( n \) corresponds to the total minima, and \( \max_i \) and \( \min_j \) represent local maximum and local minimum values, respectively, for each contraction cycle in the V-curve. Beat rate \( (br) \) is defined as the number of times a cell undergoes a contractile cycle per unit of time. We used two approaches to determine \( f \) from the curve \( d(t_k) \). In the first approach, Fourier transformation of \( d(t_k) \) exhibited dominant peaks, which corresponded to main frequencies. In the second approach, periods were selected on the \( d(t_k) \) curve in the time domain and then \( br \) was calculated as the inverse of period \( (T) \), which was defined as the time between adjacent peaks of \( d(t_k) \). After determining values of \( T \) for each adjacent peak to \( d(t_k) \), \( br \) was calculated as

\[ br = \left( \frac{1}{m} \sum_{i=1}^{m} T_i \right)^{-1}. \tag{5} \]
For periodic functions such as \(d(t_k)\), both approaches deliver the same result. The second approach offers higher flexibility when \(d(t_k)\) has high levels of noise, which may affect the calculation of \(br\) through Fourier transformation. In addition, if cell beating does not occur periodically, then multiple dominant frequencies (peaks in Fourier space) can occur. Picking periods allows the selection of individual and clearly discernible periodic motion.

To determine the time of duration of each contraction, we calculated the time at which velocity was highest in contraction and the time at which velocity was highest in relaxation.\(^\text{16}\) We then calculated the temporal distance between each adjacent maximum and minimum on the plot of \(V(t_k)\) and averaged for all contraction cycles:

\[
\hat{t} = \frac{1}{m} \sum_{i=1}^{m} \|t_k|\max_i V(t_k) - t_k|\min_i V(t_k)\|. \quad (6)
\]

The value of \(m\) captures the number of contraction cycles. Ideally, the time of each contractile cycle could be calculated from \(d(t_k)\). However, this approach is difficult because the exact beginning and end of each contraction curve are often hard to select. Determining \(\hat{t}\) has the advantage of being less biased than determining the total time of contraction because its calculation is clearly defined and always applied in the same way.

We measured two parameters of asynchronicity of contractile movement within the ROI: spatial asynchronicity \((a_B)\) of contractile movement and temporal asynchronicity \((a_D)\) of contractile movement. Spatial synchronicity occurs when distinct regions of the cell move along the same direction; temporal synchronicity occurs when all regions of the cell move at the same time.

To calculate \(a_B\), we first computed the direction of movement \((\theta)\) for every movement vector of regions \(i\) inside the ROI (Figure 1B) at each frame:

\[
\theta_i = \tan^{-1}\left(\frac{v_i}{u_i}\right), \quad (7)
\]

where \(u_i\) and \(v_i\) are the horizontal and vertical components of the displacement vectors, respectively. We expected a normal distribution of \(\theta\). If all regions within the ROI move along the same direction during contractility, then \(\theta\) will not vary among regions and the standard deviation of \(\theta_i\) will be low. However, asynchronous movement between ROI regions causes high standard deviations in \(\theta_i\). Therefore, to quantify \(a_B\), we calculated the standard deviation of \(\theta\) as a measure of the asynchronicity of displacement within the ROI:

\[
a_B = \text{mean}\left(\text{std}(\theta)(t)\right). \quad (8)
\]

To calculate \(a_D\), we first determined the mean offset time \(\delta\) between the peaks of the displacement curves of regions \((d_{\text{current}})\) in the ROI and the peaks of the mean displacement \((d_{\text{mean}})\) that occurs within the same ROI by cross-correlation between the two curves. We refined this offset result using main peaks only (Figure 6A):

\[
\delta_i = \frac{1}{N_{\text{peaks}}} \sum_{j=1}^{N_{\text{peaks}}} \delta_{i,j}, \quad (9)
\]

\[
\delta_{i,j} = t_{\max}(d_{\text{current}}) - t_{\max}(d_{\text{mean}}). \quad (10)
\]

When the timing of contractile movement of one region is synchronous with the timing of contractile movement of a reference region, values of \(\delta\) are very small. However, \(\delta\) increases if the contractile movement is asynchronous between two zones in the ROI (Figure 6C). Therefore, \(a_D\) was obtained from the standard deviation of \(\delta\) between regions that compose the ROI:
\[ a_\delta = \text{mean}([\text{std}(\delta)](t)). \quad (11) \]

If cell beating is spatially and temporally synchronous within the selected ROI, then \( a_\delta \) and \( a_\delta \) have lower values than if the beating is less synchronous.

Displacement fields for each frame were calculated with respect to a reference frame \( f_{\text{frame}_{\text{ref}}} \). The selected \( f_{\text{frame}_{\text{ref}}} \) was not necessarily the first frame of the video, but instead a frame that showed the cell in its most relaxed state. We did not control the contractile state of the cell to match the beginning of video acquisition to a relaxed state of a beating cell. Therefore, we did not know a priori the phase of the contractile cycle that the cell was in the first frame of each video. The first frame of the video could represent a cell that is fully contracted, fully relaxed, or in between those states. We selected a \( f_{\text{frame}_{\text{ref}}} \) at which the cell was in a relaxed state. The choice of \( f_{\text{frame}_{\text{ref}}} \) was critical for the shape of d-curves and V-curves, as noted in Figure 1. We automated the selection of the \( f_{\text{frame}_{\text{ref}}} \) at which the cell is fully relaxed.

In our algorithm for selecting \( f_{\text{frame}_{\text{ref}}} \), the first frame of the video was initially selected as a first possible \( f_{\text{frame}_{\text{ref}}} \) and compared with other frames to select the one that best satisfied the following criteria:

\[
\text{maximize } [m(f_{\text{frame}_{\text{ref}}}) = \Delta d \approx d_i \left(t_{f_{\text{frame}_{\text{ref}}}}\right)_{\text{max}} - d_k \left(t_{f_{\text{frame}_{\text{ref}}}}\right)_{\text{min}}], \quad (12)
\]

\[
\text{minimize } [n(f_{\text{frame}_{\text{ref}}}) = \sum_{i=1}^{N} \left(d_i \left(t_{f_{\text{frame}_{\text{ref}}}}\right)\right)]. \quad (13)
\]

To select \( f_{\text{frame}_{\text{ref}}} \), the first criterion ensures a maximal difference between the maximum and minimum points of the displacement curve. However, the solution for this criterion could be the desired \( f_{\text{frame}_{\text{ref}}} \) where the cell is in its most relaxed state, or a \( f_{\text{frame}_{\text{ref}}} \) where the cell is in its most contracted state. Reference frames between both states were excluded according to the first criterion. The second criterion selects a \( f_{\text{frame}_{\text{ref}}} \) that minimizes the area under the displacement curve, which satisfies the conditions for the d-curves presented in Figure 1. These two criteria were enough to automatically identify \( f_{\text{frame}_{\text{ref}}} \) as a frame where the cell is in its most relaxed state. In addition, all identifications of \( f_{\text{frame}_{\text{ref}}} \) were submitted to user-review of the resultant contraction d-curve. To robustly choose \( f_{\text{frame}_{\text{ref}}} \), videos had to be acquired at a speed that captured the cell at different stages of the contractile cycle. Identifying the \( f_{\text{frame}_{\text{ref}}} \) also required an image resolution (\( \mu \text{m/pixel} \)) that allowed the tracking of movement within the ROI as well as low image noise. Once \( f_{\text{frame}_{\text{ref}}} \) was selected, all displacements in regions within the ROI were calculated relative to \( f_{\text{frame}_{\text{ref}}} \).

**Traction force microscopy and phenotypes of mechanical output**

We estimated forces generated by hiPSC-CMs with a traction force microscopy algorithm. Traction force microscopy estimates forces generated by adherent cells on deformable substrates.\(^{17,18}\) This approach involves two steps: i) measuring substrate deformation induced by cell-generated traction, and ii) deriving forces from substrate deformations while considering the Young’s modulus \( (E) \) and Poisson’s ratio \( (\nu) \) of the substrate.

As noted in the fabrication section above, we dispersed fluorescent microbeads in the core of polyacrylamide hydrogels and quantified their displacement during contractions of hiPSC-CMs to track cell-induced deformations of polyacrylamide substrates. While cells were beating, we acquired videos of moving microbeads at frame rates >25 fps and submitted these videos to cross-correlation particle tracking as detailed in the beginning of the previous section. We also tracked displacement of microbeads
as defined in the previous section to determine displacement curves \(d(t)\), the maximal velocity of contraction \(V_C\), the maximal velocity of relaxation \(V_R\), the beat rate \(br\), and the time between each adjacent maximum and minimum on the velocity plot \(\hat{E}\) (Figure 1).

After quantifying cell-induced microbead displacements in hydrogels, we estimated the traction stresses \(\sigma\) associated with each displacement vector of the surface and calculated the force \(f\) for each stress vector. We then calculated the absolute value \(|F|\) of \(\sigma\) for each pixel, which has a positive value independently of its orientation or coordinates. We summed the values of \(F = \sum F\) to calculate the total amount of force that each cell can generate on its extracellular environment during each contractile cycle.\(^6\)

As we plotted \(\sum F\) as a function of time, we also plotted contractile power \(P\), which was calculated by multiplying \(\sum F\) by the velocity of microbead movement at each time point captured by a video frame. From the curve \(P(t)\), we calculated the maximal power of contraction \(P_C\) and the maximal power of relaxation \(P_R\) (Figure 1).

After determining the displacements of moving microbeads from the videos, we estimated \(\sum F\) from these cell-induced displacements with traction force microscopy.\(^{19}\) The continuum-mechanics equations for linear elastic materials are described through force equilibrium conditions,

\[
\sigma_{ij,j} + f_i = 0, \quad (14)
\]

the material constitutive relations,

\[
\sigma_{ij} = \frac{E}{1+\nu} \left[ \epsilon_{ij} + \frac{\nu}{1-2\nu} \delta_{kk} \delta_{ij} \right], \quad (15)
\]

and kinematic equations,

\[
\epsilon_{ij} = \frac{1}{2} \left( u_{i,j} + u_{j,i} \right), \quad (16)
\]

where \(\sigma\) is the stress tensor, \(f\) is a force of external origin, \(\epsilon\) is the linear strain tensor and \(u\) is the displacement field. \(E\) is the Young’s modulus of the polyacrylamide substrate and \(\nu\) is its Poisson’s ratio. \(E\) and \(\nu\) are constants that depend on the properties of the deformable material. For polyacrylamide substrates, \(E\) is tunable in the kPa range\(^{20}\) and \(\nu\) is \(-0.45\) for thin polyacrylamide sheets used for cell culture.\(^{21}\) Equations 14-16 correspond to 15 equations with 15 unknowns that are expressed in a condensed form using the Einstein summation convention and the Kronecker delta \(\delta_{ij}\) \((\delta_{ij}=0\ if\ i\neq j, \delta_{ij}=1\ if\ i=j\)\). Note that \(\delta_{ij}\) is not related to the variable defined in Equations 9 and 10, but uses the same notation.

These equations are valid assuming that the strains are small and linear and that the polyacrylamide substrate has homogeneous properties and behaves as an elastic solid.\(^{22}\) These assumptions satisfy the need for geometric linearity of strain and material linearity of the substrate.

By combining the governing equations, the balance of internal forces described in equation 14 can be written as a partial differential equation for the displacement vector field:

\[
\frac{E}{2(3+\nu)} \left[ \left( u_{j,j} + u_{i,j} \right) + \frac{2\nu}{3-2\nu} u_{k,kj} \delta_{ji} \right] + f_i = 0. \quad (17)
\]

Following the same procedure as Dembo and colleagues\(^{19}\) and Landau and colleagues,\(^{23}\) while analyzing displacements of microbeads, we considered that cell-generated deformations of a polyacrylamide surface occurred in a semi-infinite elastic medium with a planar traction distribution on its surface. Specifically for a semi-infinite elastic medium bounded by a planar surface at \(z=0\), we used a derivation of the Boussinesq solution developed by Landau and Lifshits.\(^{25}\) This solution describes deformations of the
medium under the influence of a concentrated point force $F$ applied to the surface. This relationship between local displacement ($u$) and $F$ can be represented using the Green’s tensor $G$ as

$$u_i = G_{ij}(x, y, z)F_j. \quad (18)$$

We further assumed that all local displacements are in-plane, $u = (u_x, u_y)^T$, that tractions normal to the displacement plane are zero, $F = (F_x, F_y)^T$, and that $u$ is close to 0.5 for polyacrylamide hydrogels. The problem was therefore reduced to $x$ and $y$ coordinates that represent two-dimensional movement of fluorescent microbeads being deformed due to cellular traction. Under these assumptions,

$$G_{ij} = \frac{1+\nu}{\pi E} \begin{bmatrix} (1 - \nu)r^2 + \nu x^2 & -\nu xy \\ -\nu xy & (1 - \nu)r^2 + \nu y^2 \end{bmatrix}, \quad (19)$$

where $r = \sqrt{x^2 + y^2}$ and the off-diagonal elements are corrected with a minus sign. To calculate cell-generated traction forces $T(x, y)$, Equation 17 can be represented as

$$u_i = \iint G_{ij}(x - x', y - y')T_j(x', y')dx'dy'. \quad (20)$$

Equation 19 corresponds to a spatial convolution of $G$ and $T$, which Butler and colleagues first denoted as $u = G \otimes T$, and represents displacement as a function of known traction forces. To determine $T$ as a function of $u$, we inverted Equation 20, which required transformation into the Fourier space because $G$ is not diagonal. Using the convolution theorem, the problem becomes $\tilde{u}(k) = \tilde{G}(k)\tilde{T}(k)$ and the transformed matrix $\tilde{G}$ is expressed as

$$\tilde{G}_{ij} = \frac{1+\nu}{\pi E} \frac{2\pi}{k^2} \begin{bmatrix} (1 - \nu)k_x^2 + \nu k_y^2 & \nu k_x k_y \\ \nu k_x k_y & (1 - \nu)k_y^2 + \nu k_x^2 \end{bmatrix}, \quad (21)$$

where $k = \sqrt{k_x^2 + k_y^2}$ and $k_i$ represent wave vectors.

We then computed traction forces through the inverse Fourier transformation,

$$T = \mathcal{F}^{-1}\{\tilde{G}^{-1}\tilde{u}\}. \quad (22)$$

As detailed by Butler and colleagues, to solve this equation we solved the Nyquist frequency limitation by setting the off-diagonal elements of Equation 20 to 0 if at a Nyquist frequency in $x$-axis or $y$-axis. We also filtered the displacement values resultant from noise while calculating traction forces, as previously demonstrated by Schwarz and colleagues. In summary, to filter noise without altering signal, we achieved the smoothing with zero-order Tikhonov regularization, which was initially adapted by Sabass and colleagues while solving this Fourier transformation problem. Sabass and colleagues altered Equation 21 into

$$T = \mathcal{F}^{-1}\left\{(\tilde{G}^T\tilde{G} + \lambda^2\tilde{H})^{-1}\tilde{G}^T\tilde{u}\right\}. \quad (23)$$

The regularization parameter $\lambda$ determines the amount of the solution that originates from the regularization parameter relative to the data. $\tilde{H}$ corresponds to the identity $I_2$ for a zero-order regularization.

In our MATLAB-based graphical user interfaces (GUIs: see information bellow on to use) we implemented two traction force microscopy approaches to analyze our videos of moving microbeads:
constrained and unconstrained traction force microscopy. User tutorials on how to use the graphical user interfaces are presented below. These approaches were initially developed to quantify the forces of cell adhesion to deformable substrates. Butler and colleagues have shown that defining the deformed region of the gel is key for quantifying cell adhesion forces. To exclude erroneous solutions and the effect of noise, they implemented a constrained approach in which generated forces are restricted to the area occupied by the cell. The opposite strategy is an unconstrained approach in which tractions outside of the area occupied by the cell are also considered. To measure contractile forces generated by hiPSC-CMs, we chose to do constrained or unconstrained Fourier-based traction force microscopy. These methods generate maps of surface stresses ($\sigma$) that are converted to absolute values of traction forces ($F$); we sum the values of $F$ ($\sum F$) by integrating all values of $F$ over the respective areas where cells generate contractile forces. The constrained approach yields a map of cell-generated tractions within the ROI defined by the cell borders (Figure 1B), while the unconstrained approach results from the direct conversion of force from displacement as described above. As shown in Online Figure XIII, constrained analysis computationally translates all tractions back to the area occupied by the cell, while with unconstrained analysis one obtains a more realistic translation of the contractile activity of hiPSC-CMs into tractions on the substrate. Here, we used unconstrained traction force microscopy, as further detailed ahead.

Conversion of $\sigma$ to $F$ was carried out for each quadratic element of the traction grid that results from submitting videos of moving microbeads to the image analysis routine that defines traction force microscopy. We multiplied $\sigma$ by the area of each respective grid element. For constrained force calculation, we integrated $F$ within the ROI defined by the cell borders. For unconstrained measurements, we calculated an extended ellipse with the same center of mass as the ROI (Figure 1E) and integrated $F$ within the region delimited by this extended ellipse to determine $\sum F$. This approach allows the analysis of one cell at a time within a video of multiple cells in an array; it also does not quantify noise in regions of the substrate that are far from the cell. The area of the extended ellipse relates to the area of the ROI as

$$A_{\text{ellipse}} = n.A_{\text{ROI}}.$$  \hspace{1cm} (24)

To calculate the extended ellipse, we set the constant $n$ to values between 2 and 3 and set the orientation of the major axis ($a$) and that of the minor axis ($b$) of the ellipse to match the orientation of the major and minor axes of the ROI (Figure 1E), respectively. Therefore,

$$a_{\text{ell}} = \sqrt{n}.a_{\text{ROI}},$$  \hspace{1cm} (25)

and

$$b_{\text{ell}} = \sqrt{n}.b_{\text{ROI}}.$$  \hspace{1cm} (26)

We obtained plots of $\sum F$ as a function of time from constrained and unconstrained Fourier-based traction force microscopy approaches applied to displacement maps of microbeads calculated from videos of moving microbeads. Displacement maps of microbeads were determined for each frame of a video relative to a frame of a video.

As detailed above, we selected $\text{frame}_{\text{ref}}$ to correspond to the relaxed state of a beating cell. We used the criteria defined by Equations 12 and 13, but applied them to videos of moving microbeads instead of bright-field videos of beating cells. The inputs for traction force microscopy analysis were displacement fields, hydrogel material stiffness $E$, and the Poisson ratio $\nu$ (Equations 14, 16, 18, and 20). We assumed our substrate had a Poisson ratio of 0.45 and a material stiffness of 10 kPa.
Unconstrained Traction Force Microscopy

In the unconstrained analysis, we applied a Fourier transform to each displacement map. Then, for each wave number, we set tractions at $f=0$ to 0 (Equation 13), computed $G$ according to Equation 20, and set the diagonal elements to 0 at Nyquist frequency. We considered regularization as defined in Equation 23. We calculated the regularization parameter $\lambda$ for the first frame of displacing microbeads in a video using the Regutools toolbox in MATLAB. Because noise does not vary within a video, we applied this $\lambda$ to the analysis of subsequent frames. We calculated an independent value of $\lambda$ for each video analyzed. After calculating stress values in the Fourier space for each pixel, we transformed stresses back to the real space and obtained a map of stresses for each frame.

Accurately calculating $\lambda$ is key for generating reliable solutions because Equation 23 represents an ill-posed problem in which arbitrarily small perturbations of input data can lead to arbitrarily large perturbations of the solution. Calculation of $\lambda$ via the Regutools toolbox solves an ill-posed problem defined as $Ax = b$ that satisfies the following criteria:

a. the singular values of $A$ tend to zero, and

b. the ratio between the smallest non-zero values of $A$ is large.

A side constraint ($\Omega(x)$) was introduced to minimize the norm $\|Ax - b\|$ while minimizing $\Omega(x)$. In the Tikhonov regularization approach, $\lambda$ represents the weighing between the data and $\Omega(x)$:

$$\min_x \|Ax - b\|^2 + \lambda^2 \Omega(x)^2.$$ (27)

Large values of $\lambda$ cause an excessive level of smoothing, while small $\lambda$ values increase the weight of noise pronounced in $Ax = b$. The ability of this approach to calculate a suitable $\lambda$ with the L-curve criterion as been validated.

Constrained Traction Force Microscopy

In our constrained traction force microscopy analysis, we required the same inputs that were used for the unconstrained analysis as well as information about the ROI that limits the boundaries of the cell within the frames of moving microbeads. We first calculated stresses as detailed for the unconstrained calculation and defined a new traction field by setting the tractions outside of the ROI to zero. We then calculated the displacement field that corresponds to this new traction field and replaced experimental values of displacement inside the ROI by the calculated displacement values. We iterated the calculation of stress from the displacements within the ROI to calculate new displacement values until we achieved stable values of stress within the ROI. The resulting stress values were then converted to force. However, the estimation of force via the constrained traction force microscopy approach is very susceptible to noise because high noise leads to large force values at the cell boundary.

Sarcomere length in patterned hiPSC-CMs

We analyzed videos of beating patterned hiPSC-CMs with LifeAct-labeled myofibrils to quantify the organization and dynamics of sarcomeres along myofibrils. Sarcomere shortening and movement during the contractile cycle was determined by analyzing how the size of all labeled sarcomeres in a single hiPSC-CM varies during each contractile cycle. The minimal length that separates two proximal Z-lines defines sarcomere size. LifeAct labels actin between Z-lines, which correspond to dark lines in LifeAct-labeled myofibrils. Therefore, the minimal distance between adjacent dark lines in LifeAct-labeled myofibrils defines sarcomere size. We used four approaches to quantify sarcomere size along LifeAct-labeled myofibrils from frames of single beating hiPSC-CMs (Online Figure IV); we determined that the second approach applied below performed better in determining accurate values of sarcomere length and in analyzing videos with minimal user intervention.
First Approach

The first approach (Online Figure IV) is the current state of the art for characterizing sarcomere organization from immunocytochemically labeled sarcomeres, involves the skeletonization of LifeAct-labeled regions, and was based on work initially developed by Kuo and colleagues. As detailed by Hong and colleagues, we used a fingerprint enhancement algorithm available online to optimize the quality of the skeleton obtained from frames of Life-Act labeled myofibrils. The input of the algorithm was a set of frames of a video of moving labeled sarcomeres. For each frame, the algorithm identified ridge-like regions using the ridgesgment tool. Ridge orientation was determined with the ridgeorient tool and sarcomeres were defined to be perpendicular to the orientation of adjacent myofibrils. The orientation map was rotated by $\pi/2$ and restricted to $[0; \pi]$ because Z-lines are perpendicular relative to the orientation of myofibril direction and because it is irrelevant whether the detected angle of myofibril orientation is $\alpha^\circ$ or $\alpha-180^\circ$. Then, ridge frequencies across the image were determined with the ridgefreq tool and the ridgefilter tool enhanced the ridge pattern with signal filtering, yielding a skeletonized image of myofibrils for each frame. All tools were downloaded from Peter’s Functions for Computer Vision. After obtaining a skeletonized image of myofibrils, we determined the average sarcomere length using a radial Fourier transform and selecting the dominant frequency, as described elsewhere. Briefly, we summed the radial profiles of the Fourier-transformed skeletonized image to remove user bias in selecting the main orientation of myofibrils and because we knew a priori that the orientation of sarcomeres in patterned hiPSC-CMs is not strictly perpendicular to the cell’s main axis. This summation of radial profiles leads to a one-dimensional curve ($f(\omega)$) that is normalized to ensure that the integral over all frequencies equals 1. We then considered $f(\omega)$ to result from the combination of a periodic part ($\Gamma_p(\omega)$), which contains information on the periodicity of sarcomeres, with an aperiodic part ($\Gamma_{AP}(\omega)$), which captures artifacts from imperfect skeletonization:

$$\Gamma_p(\omega) = \sum_{k=1}^{5} a_k e^{\left(\frac{\omega-k\omega_0}{\delta_k}\right)^2},$$

$$\Gamma_{AP}(\omega) = a + b e^{(-c\omega)} ,$$

where $\Gamma_p$ was approximated by a series of five Gaussian peaks that occur at the mean sarcomere frequency. Least-square fitting was then applied to estimate the parameters $a$, $b$, $c$, $\omega_0$, $a_k$, and $\delta_k$. The area under $\Gamma_p$ was also registered as a measure of sarcomere organization. A higher area under the major frequency component indicates that sarcomeres are more periodically organized. With this approach, the mean Z-line frequency ($r_0$) was determined by the frequency parameter ($\omega_0 = 1/r_0$).

In detail, the algorithm that calculated the main frequency from orientation-averaged Fourier transforms used a sarcomere skeleton as input; the output was the average sarcomere length. Each rectangular frame was transformed into a square image by adding zero values to the shorter side of the rectangular frame until each side had the same size. The resulting square image was then divided into $n$ angles. The skeleton image was rotated for each angle and 1D-Fourier transform was carried out along the x direction for each angle of image rotation. The Fourier profiles of each angle were then summed. The radial amplitude defined by $\Gamma_p$ was summed with the inspect tool and $\Gamma_p$ was normalized to yield a curve with a total area of 1. We considered sarcomere lengths in the 2-μm range. The maximum peak of $\Gamma_p$ was determined within this sarcomere range by first fitting a $p$-th order polynomial to $\Gamma_p$ and guessing the frequency peak $\omega_0$ as the maximum point of $\Gamma_p$ within a frequency range of $[0.7, \omega_0; 1.3, \omega_0]$. The mean sarcomere length was then computed as the inverse of the dominant frequency peak $r_0$.

Second Approach

The second approach (Online Figure IV) is a novel method that we developed to determine sarcomere lengths in each frame without curve-fitting procedures or radial Fourier transforms. The approach consists
of automatically measuring the length of the segment between adjacent Z-lines that is parallel to the direction of myofibril alignment (Online Figure XIV). The algorithm for this approach measured the length from Z-line \( i \) to Z-line \( i + 1 \) using information on myofibril orientation in the region around Z-line \( i \) and Z-line \( i + 1 \) and considering a skeleton of sarcomeres as described above for the first approach. The algorithm developed a map of all points that compose the sarcomere skeleton; each point was taken once as a starting location of a path along the direction of myofibril orientation that stopped when another Z-line in the skeleton was reached (Online Figure XIV). The length between adjacent Z-lines was therefore defined as the Euclidean distance between the start and end points calculated with this method (Online Figure XIV). The path was calculated pixel by pixel within the skeletonized image. Within a path composed of pixels in a line and for each new pixel of the path, the algorithm evaluated what the other pixel of the path was based on the local orientation of the myofibril (Online Figure XIV). For pixels that were starting points or pixels already in a path, the local orientation angle of myofibrils was taken as the deciding factor to determine the next path pixel. In the algorithm, we established that the \( y \) direction of the path could be chosen freely, but only pixels in the \( +x \) direction could be candidates for the next element of the path (Online Figure XIV). We set this definition because every point composing the skeleton was considered as a starting point and because orientations of myofibrils are in the \([0, \pi]\) range.

In relation to a known point of a path, the next neighboring pixel to be included could be the pixel on the right, on the top, on the bottom, on the top right, or on the bottom right (Online Figure XIV B). Given this condition, a 10° angle or a 0° angle of known myofibril orientation lead to the same decision for the next pixel to include in the path: the pixel on the right. We estimated the local orientation of myofibrils through the MATLAB-written code RIDGEORIENT,\(^{11}\) which indicates the principle ridge direction through local gradient variations. By definition, the gradient is tangential to the main orientation.\(^{33}\)

The global orientation of a myofibril between Z-lines was also considered when deciding the next pixel in the path between Z-lines (Online Figure XIV) because small angles can add up during the extending of the line defined by the path and better reveal the real myofibril orientation. For example, starting at a pixel on a Z-line \((x_1, y_1)\) with a local orientation angle \(\theta_1 = 10^\circ\), the next pixel would have to be \((x_1 + 1, y_1)\). If \(\theta_2 = 10^\circ\), then the next pixel would again be to the right, adding \((x_1 + 2, y_1)\) to the path. If \(\theta_3 = 15^\circ\) and the orientation angles of the previous path elements are ignored, then the next pixel to be included in the path should be \((x_1 + 3, y_1)\). However, if the angles of local orientation of all path elements added up to \(\theta_t = \sum^t \theta_i = 35^\circ\), then the correct solution would be to add the pixel on the top right side of the last known path element \((x_1 + 3, y_1 + 1)\). If \(\theta_4 = 10^\circ\) after adding orientation angles, then the next pixel to be added to the path would be \((x_1 + 4, y_1 + 1)\). Online Figure XIV illustrates how this algorithm chooses the path that determined the distance between Z-lines.

A maximal iteration number was set for determining the path between Z-lines to exclude faulty measurements due to holes in the skeleton or incoherent orientation maps. In summary, to obtain an output of sarcomere size from an input of skeletonized frames, all points of the skeleton were considered for the beginning of a path of the segment that separates Z-lines. Then, while the path was not outside the image window and was shorter than the maximal limits of sarcomere length and iteration number, the sum of local orientation angle and angle history \(\theta_i + \theta_e\) were computed. Based on the orientation values, the next pixel of each path was chosen until the path reached a Z-line and all requirements were satisfied. Once the path was determined, the Euclidian distance between the start and the end of the path corresponded to the sarcomere length, which was also related to the sarcomere orientation angle.

**Third Approach**

We developed another novel approach to quantity the dimensions of sarcomeres (Online Figure IV). However, this approach performed poorly compared to the first and second approaches. We used gradient watersheds for segmenting sarcomeres in an image and fit a rectangle to the region occupied by fluorescently labeled actin between Z-lines. Our goal was to identify sarcomeres and their dimensions. We termed this rectangle a “sarcomere box”; its sides fit the region occupied by sarcomeres between Z-lines. To delineate the space occupied by each box, we initially submitted the images of
myofibrils to two skeletonization steps: skeletonization of actin between Z-lines, which reveals structures aligned in the direction of myofibrils, and skeletonization of Z-lines, which yields images of structures aligned perpendicular to myofibril alignment. We then combined both skeletons to generate a grid of sarcomere boxes and fitted an ellipse to each box to determine the orientation of each sarcomere within the cell. We used the information on sarcomere orientation and the dimensions of the sarcomere box to calculate sarcomere length.

We transformed greyscale frames of LifeAct-labeled myofibrils into three-dimensional topographical maps in which the greyscale intensity of each pixel represents an altitude value. We used the gradient map in this approach because intensity gradients are high at the borders of Z-lines. The frames were binarized to separate and identify Z-lines before watershed segmentation was applied. We also used the ridge-enhancing algorithm detailed in the description of the first approach because it considerably improved the quality of segmentation. This step is especially necessary for frames with inconsistent or uneven fluorescence distribution and for sequences of frames in which intensity values are time-dependent due to photobleaching. After segmentation of sarcomeres, myofibril orientation and local frequencies were estimated with Fourier analysis as detailed for the first approach. Then, we used the knowledge that the orientation of Z-lines is perpendicular to the orientation of myofibrils to apply a second type of ridge-enhancing routine. A sarcomere map was obtained by combining both of these routines. We fitted a sarcomere box to the space between Z-lines by using information on myofibril orientation. We first fitted an ellipse to the region occupied by each sarcomere, which we geometrically characterized with a major and a minor axis as well as with an orientation for each of the two axes. These axes coincide with two levels of sarcomere orientation: Z-line orientation and myofibril orientation. Within a sarcomere, actin is oriented perpendicular to Z-lines, matching the orientation of the main axis or major axis of the sarcomere box. We computed the length of a sarcomere by fitting a rectangle to the sarcomere space between detected Z-lines and oriented in the direction of myofibril alignment. We then fitted an ellipse to this rectangle by using its geometrical definition to match the dimensions of the box that delimits the sarcomere space.

Myofibril orientation was rotated by $\pi/2$ if the orientation of the main axis of the fitted ellipses was along the direction of Z-lines. We defined the ellipse in cylindrical coordinates to facilitate this task:

$$r(\alpha) = \frac{ab}{\sqrt{(b\cos\alpha)^2+(a\sin\alpha)^2}},$$  \hspace{1cm} (30)

where $\alpha$ is the angle around the center of the ellipse, $r$ is the distance between the center and the ellipse line, $a$ is the major axis, and $b$ is the minor axis. After calculating the correct orientation of the sarcomere region, we ensured that the dimensions of the sarcomere box were correct by using the criterion of area correspondence between the original segment and the box fit. The correct rectangular dimensions of sarcomere boxes were found by requesting area correspondence between the fitted ellipse and the rectangle with the sarcomere dimensions to be determined. The correct sarcomere dimensions $w_{new}$ and $h_{new}$ were obtained by demanding equal area between the rectangle that fits the sarcomere with area $A_2$ and the initial ellipse with area $A_1$. A correction factor $c$,

$$c = \frac{A_1}{A_2} = \frac{\pi}{4},$$  \hspace{1cm} (31)

was introduced to compensate for the fact that $A_1$ corresponds to the area of an ellipse. Claiming equal area yields

$$h_{new}, w_{new} = A_2 \Leftrightarrow \frac{h}{h_{new}} = \frac{w}{w_{new}}.$$  \hspace{1cm} (32)

Given these conditions, the dimensions for each sarcomere box were calculated as
We now summarize the algorithm that we developed for this approach. The input consisted of frames of labeled sarcomeres and maximal and minimal values of sizes of the sarcomere. To obtain the sarcomere length distribution, we first performed Z-line skeletonization and repeated the skeletonization routine to delineate actin bundles between Z-lines. We combined both skeletons from the previous steps into a new skeleton and closed small holes to perform the watershed transform. We finally calculated the rectangular dimensions of each sarcomere by fitting an ellipse. The orientation angle of the ellipse and the values of its major and minor axes were used to determine sarcomere dimensions. We discarded sarcomeres with calculated lengths that did not match the range of known sarcomere lengths. The sarcomere rectangle was rotated to the correct orientation and the value of the calculated length was added to the distribution vector for each sarcomere.

**Fourth Approach**

The fourth approach that we tested (Online Figure IV) was reported by Bray and colleagues. This approach consists of drawing line scans along myofibrils, plotting the intensity profile along those lines, and measuring the length between bands that correspond to Z-lines.

**Application of the Second Approach to the Current Investigation**

The second approach performed better in determining accurate values of sarcomere length and in processing videos with minimal user intervention for analyzing sarcomere lengths in videos of LifeAct-labeled beating cells. This approach was applied to frames in a video to determine how the parameters measured via traction force microscopy or from cell movement related to sarcomere size, movement, and orientation during the contractile cycle. The first, second and third approaches required successful skeletonization of each frame, which may not be perfect due to noise or inconsistent illumination. To quantitatively compare skeletons from different frames in a video, we also generated a master skeleton based on the skeletons generated from all frames. We obtained \( N - 1 \) pseudoskeletons and one reference skeleton from all video frames. Next, we used a threshold to determine the certainty that a pixel is part of the master skeleton. For example, a threshold of 0.6 leads to a master skeleton with ridges in which 60% of pseudoskeletons from all frames displayed ridges. For processing each video, we first skeletonized each frame into ridges, chose frame \( \text{ref} \) and calculated the displacement of sarcomeres during contractions. We used this displacement information to calculate a pseudo-reference skeleton for each frame. Then, we integrated pseudo-reference skeletons into the master skeleton and set a threshold for confidence to calculate the final skeletons for each frame \( i \) using the same displacement results. Displacements were calculated with the cross-correlation algorithm Ncorr, a cross-correlation approach with high performance in characterizing movement (Online Figure V). From this analysis, we obtained all parameters associated with movement on videos of cells imaged with bright-field microscopy.
Online Table I
Contractile parameters obtained for the cell in Figure 3 (isoproterenol) and the cell in Figure 4 (omecamtiv mecarbil) from traction force microscopy (TFM), from the analysis of the movement of myofibrils labeled with LifeAct, and from the analysis of cell movement from bright-field videos.

<table>
<thead>
<tr>
<th>Isoproterenol TFM</th>
<th>( \Sigma F_{\text{max}} ) (( \mu \text{N} ))</th>
<th>( V_R ) (( \mu \text{m/s} ))</th>
<th>( V_C ) (( \mu \text{m/s} ))</th>
<th>( P_R ) (pW)</th>
<th>( P_C ) (pW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ( \mu \text{M} )</td>
<td>0.53</td>
<td>1.05</td>
<td>1.68</td>
<td>0.28</td>
<td>0.7</td>
</tr>
<tr>
<td>0.1 ( \mu \text{M} )</td>
<td>0.58</td>
<td>0.85</td>
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Online Figure I. Overview of our experimental process, from micropatterning of hiPSC-CMs to obtaining parameters of their mechanical output.

Our integrated computational approach analyzes videos acquired via microscopy and derives parameters of cell mechanical output. We calculate parameters that characterize contractile and kinetic properties of the mechanical output of cells, as well as parameters of sarcomere activity and synchronicity of cell beating. We provide a semi-automatic platform for data processing, analysis of results and quantification of parameters of mechanical output. Computation power is the only limit for the level of throughput of this platform. Cell micropatterning, myofibril labeling and video acquisition were done manually and represent the current bottleneck for using the complete assay in high throughput.
Online Figure II

Online Figure II. Array of single hiPSC-CMs micropatterned on the surface of a polyacrylamide substrate. The picture shows the surface of a hydrogel device with hiPSC-CMs and was acquired with bright-field microscopy. Hydrogels were also bonded to the surface of a glass coverslip. The surface of hydrogels contained Matrigel micropatterns with a rectangular shape as defined in the Online Methods section. Micropatterns are separated from each other at a distance of 50 μm. Single live hiPSC-CMs that attached to these micropatterns systematically had an elongated shape. Scale bar: 500 μm.
Online Figure III. Derivation of some of the parameters noted in Online Figure I from curves calculated from the analysis of cellular-induced movement (Figure 1).

Peak average displacement ($d_{\text{max}}$), beat rate ($br$), peak velocity of contraction ($V_c$), peak velocity of relaxation ($V_R$), peak force ($\Sigma F_{\text{max}}$), peak power of contraction ($P_c$), and peak power of relaxation ($P_R$) are automatically derived from cross-correlation analysis of movement detected via traction force microscopy.
Online Figure IV. Testing strategies to calculate sarcomere length (sl) from an image of fluorescently labeled myofibrils in a single micropatterned hiPSC-CM.

Sarcomeres were skeletonized from the image of labeled myofibrils for the first, second, and third approaches. In the first approach, the dominant sarcomere size was calculated from the two-dimensional spatial frequency plot that results from the Fourier transform of the skeletonized image. In the second approach, average sarcomere length was determined by measuring the length between Z-lines in the image of skeletonized sarcomeres. We obtained heat maps of the distribution of sarcomere length. For the third approach, watershed segmentation was used to isolate the space between Z-lines; we calculated sarcomere length from the main axis of the region occupied by a sarcomere. In the fourth approach, lines were randomly drawn along myofibrils, and we calculated sarcomere length from the intensity profiles of these lines.
We obtained d-curves from brightfield video (Online Movie IV) of a beating micropatterned hiPSC-CM. We used three cross-correlation approaches: Ncorr\textsuperscript{15}, PIVlab\textsuperscript{13}, and ImageJ PIV\textsuperscript{14}. These analyses were repeated for the same video after decreasing the image resolution of its frames and adding image noise.
Online Figure VI. Isoproterenol-induced variations calculated with traction force microscopy (A-K) and cross-correlation of bright-field videos (L-O).

Parameters of mechanical output were determined from videos of moving microbeads with traction force microscopy for six micropatterned hiPSC-CMs at two concentrations of isoproterenol. We then calculated the variation of each parameter relative to its value when isoproterenol was absent from the extracellular milieu. 

A, Representative $F$-curve estimated for a micropatterned hiPSC-CM before adding isoproterenol (ISO). B, $F$-curve after exposing the cell to 0.1 μM isoproterenol (ISO). C, $F$-curve after adding isoproterenol (ISO) to achieve a concentration of 1 μM.

D-K, Variations in parameters of mechanical output. D, Variation in $d_{\text{max}}$. E, Variation in $V_{c}$. F, Variation in $V_{p}$. G, Variation in $\dot{\epsilon}$. H, Variation in $b_{\dot{e}}$. I, Variation in $F_{\text{c}}$. J, Variation in $P_{R}$. K, Variation in $P_{R}$. L-O, Contractile displacement ($d$) was analyzed via cross-correlation within a region of interest (ROI) delimited by the contour of the area of adhesion of six single beating hiPSC-CMs (Figure 1B) before and after adding isoproterenol at concentrations of 0.1 μM and 1 μM. L, Variation in $d$ within the ROI. M, Variation in $V_{C}$ for each contractile cycle within the ROI. N, Variation in $V_{R}$ for each contractile cycle within the ROI. O, Variation in $\dot{\epsilon}$. *P < 0.05, **P<0.01 and ***P<0.005 by the unpaired Wilcoxon–Mann–Whitney rank-sum test. Error bars represent the standard error of the mean; n.s., not significant.
Online Figure VII. Variations in parameters of mechanical output induced by omecamtiv mecarbil.

We estimated parameters of mechanical output from traction force microscopy of videos of microbeads moving due to tractions generated by six contractile micropatterned hiPSC-CMs. We calculated the variation in the values of these parameters after adding 0.1 μM or 10 nM omecamtiv mecarbil. A, Variation in \( d_{\text{max}} \) of microbeads. B, Variation in \( V_{c} \) of microbeads. C, Variation in \( V_{r} \). D, Variation in \( f \). E, Variation in \( b_r \). F, Variation in \( \Sigma F_{\text{max}} \). G, Variation in \( P_c \). H, Variation in \( P_r \). *P<0.01 by unpaired Wilcoxon–Mann–Whitney rank-sum test. Error bars represent the standard error of the mean; n.s., not significant. I, Representative chronic (5 min) change in the \( F \)-curve of a beating micropatterned hiPSC-CM after adding 0.1 μM omecamtiv mecarbil (OM). J, Change in the \( F \)-curve of a beating micropatterned hiPSC-CM detected within 10 s of adding 0.1 μM omecamtiv mecarbil (OM).
Online Figure VIII

Online Figure VIII. Detection of sarcomere damage in micropatterned hiPSC-CMs after adding omecamtiv mecarbil. We observed damaged myofibrils (green arrows) after incubating cells for 5 min in omecamtiv mecarbil at A, 1 μM, B, 0.1 μM, or C, 10 nM. Scale bar: 20 μm.
Online Figure IX

Online Figure IX. Detailed calculation of sarcomere length (sl) from videos of beating micropatterned hiPSC-CMs.

A, Beating micropatterned hiPSC-CM with fluorescently labeled myofibrils (Online Movie XI). B, Skeletonization of sarcomeres (Online Movie XII) for each frame of a video of a beating micropatterned hiPSC-CM with labeled myofibrils. C, Heat map of sarcomere length within a micropatterned hiPSC-CM calculated for one frame of video (Online Movie XIII). D, Average sarcomere length calculated for each frame and plotted as a function of time. We selected maximal average sizes (red dots) and minimal average sizes (green squares) from these curves to calculate sarcomere shortening. Scale bar: 15 μm.
Online Figure X. Generation and characterization of knockout MYBPC3 hiPSC-CMs.
A, Schematic diagram of the targeting strategy to generate knockout MYBPC3 hiPSC-CMs. The targeting construct contains ~500 bp homology arms flanking the TALEN cut site (~20 bp downstream of ATG start site) and a CAG-driven mCherry-2A-Puro cassette for selection of stable hiPSC-CM colonies. Upon successful TALEN-assisted homologous recombination, the KO donor vector introduces an early stop codon into exon 1. B, mRNA was extracted from day-15 differentiated WT and KO hiPSC-CMs. MYBPC3 expression level was normalized against cardiac-specific transcripts TNNT2 and MYH6. Data suggest reduced expression and the complete absence of MYBPC3 mRNA in MYBPC3+/− and MYBPC3−/− hiPSC-CMs, respectively. C, Total protein was extracted from hiPSC-CMs and subjected to western blot analysis using an antibody to MYBPC3. Antibody to ACTN2 was used as an hiPSC-CM-specific loading control and GAPDH as a housekeeping control. Heterozygous KO hiPSC-CMs showed reduction in protein content and homozygous KO hiPSC-CMs showed complete absence of protein. D, Immunostaining of replated WT, MYBPC3+/− and MYBPC3−/− hiPSC-CMs with antibodies against MYBPC3 (green) and ACTN2 (red).
Online Figure XI. Using our traction force microscopy algorithm with hiPSC-CMs that were not cultured as single cells on micropatterns on hydrogel devices.

A, Bright-field image of a single unpatterned hiPSC-CM on the surface of a hydrogel device. Scale bar: 20 μm. B, Bright-field image of a colony of hiPSC-CMs on the surface of a hydrogel device. Scale bar: 60 μm. C and D, Heat maps of traction stresses (scale in Pa) generated during the contractile cycle of the single unpattered hiPSC-CM (C) and of the unpatterned hiPSC-CM colony (D).
Online Figure XII. Expression of LifeAct as a function of time after infection of hPSC-CMs within a colony of cells on a matrigel pattern. Some cells expressing LifeAct (red) are observed after 2 days of infection. Higher number of LifeAct-expressing cells is observed at 1 week after infection. The number of infected cells does not change at 1.5 weeks.
Online Figure XIII. Traction force microscopy-based approaches to estimate forces generated by micropatterned hiPSC-CMs. A, Cell borders defined a region of interest (ROI). B, Displacement of microbeads in the substrate was quantified with the cross-correlation algorithm Ncorr. C, Unconstrained traction force microscopy estimates tractions (σ) directly from the displacement of microbeads. We derived force only from the tractions in the space enclosed within the green ellipse, which was calculated from the dimensions of the ROI (Online Methods). D, Constrained traction force microscopy estimates force generated within the ROI through an approach that initially considers the results from the unconstrained analysis. After using this approach, we detected tractions in regions that do not coincide with cell-generated deformations on the substrate (white arrows). Scale bar: 15 μm.
Online Figure XIV. Automated calculation of sarcomere length (sl) from the distance between Z-lines. This method results in the approach presented in Online Figure IV (second approach) and Online Figure IX; this method was used for calculating sl and sarcomere shortening. A, Calculation of sarcomere length. A line is drawn between pairs of Z-lines in a frame that was skeletonized. This procedure starts on the Z-line and ends on the Z-line to its right. Drawing considers the orientation of the myofibril going through each pair of Z-lines. B, Drawing the line between Z-lines. Considering the current point as the leading pixel of the line being drawn, there are five options for continuing the line: top pixel (t), top-right pixel (tr), right pixel (r), bottom-right pixel (br), and bottom pixel (b). The automatic decision on the choice of the pixel to extend the line is based on the history of local myofibril orientation around the previous pixels of the line. The resulting line should align along the average orientation the myofibril between the pair of Z-lines being processed. If these criteria are not met, then another decision will be made.
LEGENDS FOR VIDEO FILES

**Online Movie I** Single beating micropatterned hiPSC-CM imaged with bright-field microscopy. Video was acquired at a speed of 31.2 fps.

**Online Movie II** Moving fluorescent microbeads embedded in the polyacrylamide substrate underneath the micropatterned hiPSC-CM presented in Online Movie I. Video was acquired at a speed of 31.2 fps.

**Online Movie III** Moving fluorescently labeled myofibrils in the micropatterned hiPSC-CM presented in Online Movie I. Video was acquired at a speed of 10.6 fps.

**Online Movie IV** Single beating micropatterned hiPSC-CM imaged with bright-field and used to test different cross-correlation techniques as presented in Online Figure V. Video was acquired at a speed of 29 fps.

**Online Movie V** Fluorescently labeled myofibrils in a beating micropatterned hiPSC-CM before being incubated in isoproterenol as detailed in Figure 3. Video was acquired at a speed of 10 fps.

**Online Movie VI** Fluorescently labeled myofibrils in the micropatterned hiPSC-CM presented in Online Movie V after being incubated in 0.1 μM of isoproterenol. Video was acquired at a speed of 13.2 fps.

**Online Movie VII** Fluorescently labeled myofibrils in the micropatterned hiPSC-CM presented in Online Movie V after being incubated in 1 μM of isoproterenol. Video was acquired at a speed of 13.2 fps.

**Online Movie VIII** Fluorescently labeled myofibrils in a beating micropatterned hiPSC-CM before being incubated in omecamtiv mecarbil as detailed in Figure 4. Video was acquired at a speed of 6 fps.

**Online Movie IX** Moving fluorescently labeled myofibrils in the micropatterned hiPSC-CM presented in Online Movie VIII right after incubation in 0.1 μM of omecamtiv mecarbil. Video was acquired at a speed of 6 fps.

**Online Movie X** Moving fluorescently labeled myofibrils in the micropatterned hiPSC-CM presented in Online Movie VIII after 2 minutes of incubation in 0.1 μM of omecamtiv mecarbil. Video was acquired at a speed of 6 fps.

**Online Movie XI** Moving fluorescently labeled myofibrils in the micropatterned hiPSC-CM presented in Online Figure IX A. Video was acquired at a speed of 7 fps.

**Online Movie XII** Moving skeleton of sarcomeres presented in Online Figure IX B and calculated from labeled myofibrils as presented in Online Movie XI.

**Online Movie XIII** Varying heat map of sarcomere lengths as presented in Online Figure IX C and calculated from sarcomere skeletons presented in Online Movie XII. Darker red regions represent shorter sarcomere lengths and lighter yellow regions represent longer sarcomere lengths.
SUPPLEMENTAL REFERENCES


TUTORIALS FOR USING GRAPHICAL USER INTERFACES

We developed graphical user interfaces (GUIs) to be used in MATLAB (R2014b version, MathWorks) for applying the presented methodological approaches.

The following section consists of 3 tutorials detailing how to use each GUI to respectively quantify the mechanical output from bright-field videos of moving single cells (Tutorial Brightfield GUI), from fluorescent videos of moving sarcomeres (Tutorial Lifeact GUI) and from fluorescent videos of moving microbeads (Tutorial TFM GUI). The codes that compose each GUI are open source and were placed in a public online repository for download: [http://doi.org/10.5281/zenodo.495617](http://doi.org/10.5281/zenodo.495617).

If you deploy or build on these GUIs and their codes in your research, we request that you please cite this original manuscript Ribeiro et al “Multi-Imaging Method to Assay the Contractile Mechanical Output of Micropatterned Human iPSC-Derived Cardiomyocytes” in Circulation Research and the Github DOI: 10.5281/zenodo.495617.
The following document is a tutorial on our Brightfield analysis tool. It serves as an introductory getting-started guide.

Contents

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2 Tutorial 2
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2.2 Outline Window 4
2.3 Displacement Window 5
2.4 Parameter Window 6

3 Folder & Files Structure 9

1 Requirements and Comments

- The code has been tested on all versions of Matlab between R2014a and R2016a. It has been tested for Windows (7, 8, 10), OS X (10.9) and Linux (Arch). The GUI looks best on Windows, with minor scaling issues on the other platforms.

- The code uses some third-party packages, that we include in our repository. This is the complete list:
  - Ncorr\(^1\): DIC algorithms.
  - Bio-Formats toolbox\(^2\): read image and video formats.
  - xlwrite\(^3\): write results to Excel files.
  - peakdet\(^4\): determine peaks on noisy curves.
  - statusbar\(^5\) and enableDisableFig\(^6\): freeze the windows during calculations and estimate runtime.
  - freezeColors\(^7\) and COLORMAP and COLORBAR utilities\(^8\): display overlays on figures.
  - Exportfig\(^9\): flexible plot exports.

- On recent versions of Matlab, the GUI produces a variety of warnings related to packages used for blocking the interface during calculation. These can safely be ignored.

- During execution, temporary files as well as result data is written to the disk. Thus it is advisable to have at least a few hundred MB of free disk space. Also, all the functions and the file structure has to be left unaltered, and put in a place that has writing permissions.

- Windows will generally freeze during intense calculation to prevent all user input, then resume post-computationally. Calculations can however be stopped using the usual \(\text{ctrl} + \text{c}\) key combination. It is mandatory to close all windows with there respective \text{OK} buttons, not using the OS window close button.

---

\(^1\)http://ncorr.com/
\(^2\)http://www.openmicroscopy.org/site/support/bio-formats5/users/matlab/
\(^3\)http://www.mathworks.com/matlabcentral/fileexchange/38591-xlwrite--generate-xls-x--files-without-excel-on-mac-linux-win
\(^4\)http://www.billauer.co.il/peakdet.html
\(^5\)http://www.mathworks.com/matlabcentral/fileexchange/14773-statusbar
\(^6\)http://www.mathworks.com/matlabcentral/fileexchange/15895-enable-disable-figure
\(^7\)http://www.mathworks.com/matlabcentral/fileexchange/7943-freezecolors---unfreezecolors
\(^8\)http://www.mathworks.com/matlabcentral/fileexchange/24371-colormap-and-colorbar-utilities--jul-2014--
\(^9\)http://www.mathworks.com/matlabcentral/fileexchange/727-exportfig

August 28, 2016
2 Tutorial

The main window is opened by executing `bf_gui_main.m`, and changing the working directory to the current folder. Figure (1) shows the main window of the analysis.

![Main window of the analysis](image)

*Figure 1: Main window; in blue, the relevant sections of the tutorial*

The analysis is split into four parts, each of them requiring user setup and review. The order of going through these is by increasing number.

Steps which are not accessible at user's current stage are greyed out, steps which have been completed show up green.

2.1 Initialization Window

![Initialization window](image)

*Figure 2: Initialization window*

One or more videos must be added to the analysis. Videos can be in the following formats:
• *.czi video files
• *.avi video files
• *.tiff image stacks

It is also possible to indicate a folder containing *.png images. Images are read in based on the order that they are listed in the folder, so images should be in the correct order, e.g. {im_001.png, im_002.png, im_003.png,...}. Be careful to pad numbers in file names with zeros, im_11.png generally list before im_2.png.

Once multiple videos are loaded, it is possible to review and browse through them. The axes displays the first frame of the respective video, and the video information is chosen accordingly.

Videos can be deleted from the analysis list, after they have been loaded. This may be useful if a whole list has been input, but one video is not suitable for analysis.

Some information about the video is needed, in particular the framerate, the number of frames and the resolution. For identification purposes in view of post-processing, cells should also be assigned a unique and characterizing name tag. No two videos are allowed the same cell name!

Depending on the format of the videos, the metadata is accessed automatically.

• For *.czi video files: whole metadata is available.
• For *.avi video files, *.tiff image stacks, image folders: user has to input framerate and resolution, whereas the number of frames is detected.

The video information is saved in the cell-specific Excel sheet. For more information about this, see section 3 on folder and files organization.

Based on video format, panels are enabled and disabled in a smart way. For instance for videos where the framerate is already available, no user input is required, which causes the respective input boxes to be greyed.

In case that the [OK] button is activated, but important information is still missing, e.g. the conversion factor for one video has not been entered, a reminder message will pop up.
2.2 Outline Window

The cell outline should be drawn. Then a preview of the cell ratio and angle is shown to verify the selection.

For each cell, in addition to the cell as a whole, which is a mandatory step, the user can opt to additionally specify one or more regions of interest, for which parameters should also be determined.

These first two steps should be repeated for every video that has been loaded.

Closes the window.

Gives a preview of dimensions (in pixels), angle and ratio of the user-drawn outline. This is not used for any calculations, and is mainly given for information purposes.

A warning message will ask the user to provide all necessary outline, in the case where some are forgotten.
2.3 Displacement Window

**Figure 4: Displacement window**

- Displacements are computed with the Ncorr DIC code. Brightfield videos are sparse on markers for exact displacement calculation. It is absolutely essential that the computed displacements are as accurate as possible.

  For DIC, the important parameters are subset size and radius. The size parameter is a tradeoff between accuracy and time-efficiency. Ideally it would be set very low (ideally 0) to get very high resolution, whereas large values produce an excessive smoothening effect. The radius is pretty tricky to set. It should ideally be as small as possible without getting noise effects.

  To improve performance, only areas inside the cell outline are considered for displacements.

- All videos are processed with identical analysis settings. The preview gives a heatmap preview of the current frame, with a fixed pre-set axis scale and vector length. For some videos, the preview thus might not look amazing, however usually this is a scaling issue.

- Displacements are calculated with an arbitrary reference frame, the first frame of the video. For display and interpretation purposes, it is generally favourable to have a relaxed cell state as reference. This step smart-guesses the optimal reference frame.

- The reference auto-guessing usually is very reliable. However there are cases, where the prediction is not accurate, e.g. when the videos are really small, or the cell movement is particularly unsynchronized, coupled with excessive image noise. A new reference value can be selected by picking a point as reference or inputting a frame number.

  The contracted frame is not used for further calculations, so it is not necessary to change this, too. The reason the guess for the contracted frame is given is that for the unlikely case that the auto-guess failed, usually the issue can be fixed by exchanging relaxed and contracted guesses.
The previews should be visually inspected for all videos. Afterwards, displacement data has to transformed to the new reference. In addition, the synchronicity calculation is performed at this stage.

A lot of data is generated during the DIC analysis, which can optionally be stored for further processing. The options here are: saving the full displacement field in matrix form, as they are used during the analysis, and saving the preview heatmaps.

**Important Note:** Saving takes some time and should only be enabled if necessary.

The window is closed and the selected saving options are written to the disk. All Excel result files are updated with reference and contracted frame.

### 2.4 Parameter Window

![Figure 5: Parameter window](image)

**Note:** all of the following parameters can be left empty. If for instance one curve does not look good, and it is not possible to extract reliable information, just leave it out. The associated parameter will appear as NaN, which is left empty in Excel later.

The maxima and minima of the displacement and velocity curves can be estimated in an automated way. This is not always accurate, and should be reviewed and corrected.

Input the aspect ratio of patterns.
Average displacement defined for each timepoint (i.e. frame) as the mean of all displacements inside cell blob,

\[ d(t_k) = \frac{1}{N} \sum_{k}^{N} \sqrt{u_{k,x}^2 + u_{k,y}^2}. \]  

(1)

The contraction displacement then is the total distance between relaxed and contracted state, or in other words, the distance between minima and maxima on the \( d(t) \) curve,

\[ d_{\text{contr}} = \frac{1}{m} \sum_{i}^{m} \max(d(t_k)) - \frac{1}{n} \sum_{j}^{n} \min(d(t_k)). \]  

(2)

Average velocity defined for each timepoint (i.e. frame) using the mean displacements \( d(t_k) \),

\[ v(t_k) = \frac{\Delta d}{\Delta t} = \frac{d_{k+1} - d_{k-1}}{t_{k+1} - t_{k-1}}. \]  

(3)

The maximal contraction and relaxation velocities then correspond to maxima and minima of the \( v(t_k) \) curve,

\[ v_{\text{max,contr}} = \frac{1}{m} \sum_{i}^{m} \max(v(t_k)) ; \quad v_{\text{max,relax}} = \frac{1}{n} \sum_{j}^{n} \min(v(t_k)). \]  

(4)

To determine frequency of contraction, there are two possibilities.
(i) The signal can be Fourier transformed. Then the peaks correspond to main frequencies of the signal.

\[ f \text{ [Hz]} \]

\[ \cdot 10^{-6} \]

Figure 8: Fourier transformed signal

(ii) Use the definition of frequency as inverse of the period \( T = \frac{1}{f} \). Using the displacement signal \( d(t_k) \), it is easy to draw periods on the curve. For multiple contractions, the mean can then be computed,

\[ f = \left( \frac{1}{m} \sum_{i} T_i \right)^{-1}. \quad (5) \]

\[ \cdot 10^{-7} \]

\[ d(t) \text{ [m]} \]

\[ t \text{ [s]} \]

\[ 0 1 2 3 4 5 6 7 8 9 10 \]

\[ 0 1 2 3 4 5 6 7 8 9 10 \]

\[ T \]

Figure 9: Displacement plot with time period

Method (ii) is probably the safer choice, as it is quite clear what is selected, especially in cases where the curve is not really periodic.

- \( f_1 \) Displays the evolution of the mean of the standard deviation of the direction angles of displacements inside the cell (measure of spacial synchronicty). No user input required.

- \( f_4 \) Displays temporal synchronicty: heatplot/histogram of the synchronicty \( \delta \).

- \( h_4 \) It is difficult to evaluate the contraction time of one contraction, as it is hard to determine when it exactly starts and ends. However one can take a value that scales proportionally to contraction time: we take the time between a velocity maximum and minimum of a contraction cycle,

\[ \hat{t} = \frac{1}{m} \sum_{i} \left| t_k \right| \max_{i} v(t_k) - \left| t_k \right| \min_{i} v(t_k) \right|. \quad (6) \]
Figure 10: Velocity plot with $\dot{t}$

- Tag if there are double peaks.
- Tag if cell does not look useful for further analysis.
- Go through all the videos and input parameters. Also gives a preview of the current cell outline or ROI.
- Curve data and generated parameters are saved in the result file. The user can optionally select to also save curve plots. This then closes the window and concludes the analysis.

3 Folder & Files Structure

- Creation of a temporary folder for each video in the path of the Brightfield GUI:

```
Brightfield GUI Folder/
    vars_DO_NOT_DELETE/ contains temporary folders for all cells
        Experiment 1/ all data for cell 1
            image1.mat image data
            dis_pts1.mat total displacement
            piv_x1.mat x-coordinates matrix
            piv_y1.mat y-coordinates matrix
            piv_u1.mat u displacement matrix
            piv_v1.mat v displacement matrix
            ...
        Experiment 2/ all data for cell 2
            ...
        ...
```

Figure 11: Folder tree for temporary data

- Creation of a result folder for each video in the path of the video file:
Figure 12: Folder tree for result data

- For each cell video, a main Excel result file is created, which contains the microscope data, the curve data as well as the generated parameters. The file is organized into separate worksheets.
The following document is a tutorial on our Lifeact analysis tool. It serves as an introductory getting-started guide.

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  2.3 Displacement Window ............................................ 5
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1 Requirements and Comments

- The code has been tested on all versions of Matlab between R2014a and R2016a. It has been tested for Windows (7, 8, 10), OS X (10.9) and Linux (Arch). The GUI looks best on Windows, with minor scaling issues on the other platforms.

- The code uses some third-party packages, that we include in our repository. This is the complete list:
  - Ncorr\(^1\): DIC algorithms.
  - Bio-Formats toolbox\(^2\): read image and video formats.
  - xwrite\(^3\): write results to Excel files.
  - peakdet\(^4\): determine peaks on noisy curves.
  - statusbar\(^5\) and enableDisableFig\(^6\): freeze the windows during calculations and estimate runtime.
  - freezeColors\(^7\) and COLORMAP and COLORBAR utilities\(^8\): display overlays on figures.
  - Exportfig\(^9\): flexible plot exports.

- On recent versions of Matlab, the GUI produces a variety of warnings related to packages used for blocking the interface during calculation. These can safely be ignored.

- During execution, temporary files as well as result data is written to the disk. Thus it is advisable to have at least a few hundred MB of free disk space. Also, all the functions and the file structure has to be left unaltered, and put in a place that has writing permissions.

- Windows will generally freeze during intense calculation to prevent all user input, then resume post-computationally. Calculations can however be stopped using the usual \(\text{ctrl} + \text{c}\) key combination. It is mandatory to close all windows with there respective \(\text{OK}\) buttons, not using the OS window close button.

---

\(^1\)http://ncorr.com/
\(^2\)http://www.openmicroscopy.org/site/support/bio-formats5/users/matlab/
\(^3\)http://www.mathworks.com/matlabcentral/fileexchange/38591-xwrite--generate-xls-x--files-without-excel-on-mac-linux-win
\(^4\)http://www.billauer.co.il/peakdet.html
\(^5\)http://www.mathworks.com/matlabcentral/fileexchange/14773-statusbar
\(^6\)http://www.mathworks.com/matlabcentral/fileexchange/15895-enable-disable-figure
\(^7\)http://www.mathworks.com/matlabcentral/fileexchange/7943-freekolors---freakolors
\(^8\)http://www.mathworks.com/matlabcentral/fileexchange/24371-colormap-and-colorbar-utilities---jul-2014-
\(^9\)http://www.mathworks.com/matlabcentral/fileexchange/727-exportfig
2 Tutorial

The main window is opened by executing `lifeact_main.m`, and changing the working directory to the current folder. Figure 1 shows the main window of the analysis.

![Figure 1: Main window; in blue, the relevant sections of the tutorial](image)

The analysis is split into five parts, each of them requiring user setup and review. The order of progress is by increasing number.

Steps which are not accessible at user’s current stage are greyed out, steps which have been completed show up in green typeset.

2.1 Initialization Window

![Figure 2: Initialization window](image)
One or more videos must be added to the analysis. Videos can be in the following formats:

- *.czi video files
- *.avi video files
- *.tiff image stacks

It is also possible to indicate a folder containing *.png images. Images are read in based on the order that they are listed in the folder, so images should be in the correct order, e.g. {im_001.png, im_002.png, im_003.png,...}. Be careful to pad numbers in file names with zeros, im_11.png generally list before im_2.png.

Once multiple videos are loaded, it is possible to review and browse through them. The axes displays the first frame of the respective video, and the video information is chosen accordingly.

Videos can be deleted from the analysis list, after they have been loaded. This may be useful if a whole list has been input, but one video is not suitable for analysis.

Some information about the video is needed, in particular the framerate, the number of frames and the resolution. For identification purposes in view of post-processing, cells should also be assigned a unique and characterizing name tag. No two videos are allowed the same cell name!

Depending on the format of the videos, the metadata is accessed automatically.

- For *.czi video files: whole metadata is available.
- For *.avi video files, *.tiff image stacks, image folders: user has to input framerate and resolution, whereas the number of frames is detected.

The video information is saved in the cell-specific Excel sheet. For more information about this, see section 3 on folder and files organization.

Based on video format, panels are enabled and disabled in a smart way. For instance for videos where the framerate is already available, no user input is required, which causes the respective input boxes to be greyed.

In case that the [OK] button is activated, but important information is still missing, e.g. the conversion factor for one video has not been entered, a reminder message will pop up.
2.2 Outline & Concentration Window

The cell outline should be drawn. Then a preview of the cell ratio and angle is shown to verify the selection.

Sarcomere concentration, i.e. ratio of the cell that is filled with myofibrils, can be determined by changing the slider value until the desired picture is achieved. It is impossible to have a concentration bigger than 1, therefore everything outside the cell that was selected with the slider threshold is neglected. Uneven illumination can be an issue here.

For each cell, in addition to the cell as a whole, which is a mandatory step, the user can opt to additionally specify one or more regions of interest, for which parameters should also be determined.

These first three steps should be repeated for every video that has been loaded.

Closes the window.
2.3 Displacement Window

Displacements are computed with the Ncorr DIC code. Lifeact videos are sparse on markers for exact displacement calculation. It is absolutely essential that the computed displacements are as accurate as possible. For DIC, the important parameters are subset size and radius. The size parameter is a tradeoff between accuracy and time-efficiency. Ideally it would be set very low (ideally 0) to get very high resolution, whereas large values produce an excessive smoothing effect. The radius is pretty tricky to set. It should ideally be as small as possible without getting noise effects.

To improve performance, only areas inside the cell outline are considered for displacements.

**Important Note:** Also it is possible to change these parameters, it is not recommended. The optimal settings are a very small spacing value (0 or 1 ideally), as well as no post-processing.

All videos are processed with identical analysis settings. The preview gives a heatmap preview of the current frame, with a fixed pre-set axis scale and vector length. For some videos, the preview thus might not look amazing, however usually this is a scaling issue.

Displacements are calculated with an arbitrary reference frame, the first frame of the video. For display and interpretation purposes, it is generally favourable to have a relaxed cell state as reference. This step smart-guesses the optimal reference frame.

The reference auto-guessing usually is very reliable. However there are cases, where the prediction is not accurate, e.g. when the videos are really small, or the cell movement is particularly unsynchronized, coupled with excessive image noise. A new reference value can be selected by picking a point as reference or inputting a frame number.

The contracted frame is not used for further calculations, so it is not necessary to change this, too. The reason the guess for the contracted frame is given is that for the unlikely case that the auto-guess failed, usually the issue can be fixed by exchanging relaxed and contracted guesses.
The previews should be visually inspected for all videos. Afterwards, displacement data has to be transformed to the new reference. In addition, the synchronicity calculation is performed at this stage.

A lot of data is generated during the DIC analysis, which can optionally be stored for further processing. The options here are: saving the full displacement field in matrix form, as they are used during the analysis, and saving the preview heatmaps.

**Important Note:** Saving takes some time and should only be enabled if necessary.

The window is closed and the selected saving options are written to the disk. All Excel result files are updated with reference and contracted frame.

### 2.4 Sarcomere Analysis Window

Using an initial guess for sarcomere ranges, the skeleton for the first video frame is previewed. In addition, both the relaxed and contracted state are translated back to the first frame, for user evaluation if the displacement information is suitable for master skeleton analysis.

The skeletonization needs guesses for the sarcomere ranges, in order to generate valid $z$-lines. Special care has to be taken to restrict this to a useful range. For instance if sarcomere means are expected to lie around 1.3 micron, the maximum should be lower than 2.6, and the minimum larger than 0.65, in order to avoid double or half periods.

It is preferable for both accuracy and comparability to generate a single master skeleton for each cell. However, in order for this to work, displacement calculations have to be really precise. The user should review both the left and right image, and evaluate how well red and blue skeletons correspond.
Then a choice is made whether the regular or the master analysis shall be performed.

After the choice has been made for all videos in the stack, the calculation can begin. Skeleton images as well as distance heatmaps are saved to the disk (c.f. section 3). If unsure, the user should review these after calculation is done to make sure the sarcomere ranges were chosen correctly.

If the heatmaps and the skeleton images should be kept, the user can opt to do so. This also closes the window.

2.5 Parameter Window

Note: all of the following parameters can be left empty. If for instance one curve does not look good, and it is not possible to extract reliable information, just leave it out. The associated parameter will appear as NaN, which is left empty in Excel later.
The maxima and minima of the displacement and velocity curves can be estimated in an automated way. This is not always accurate, and should be reviewed and corrected.

Average displacement defined for each timepoint (i.e. frame) as the mean of all displacements inside cell blob,

\[
d(t_k) = \frac{1}{N} \sum_{k}^{N} \sqrt{u_{k,x}^2 + u_{k,y}^2}.
\]  

(1)

The contraction displacement then is the total distance between relaxed and contracted state, or in other words, the distance between minima and maxima on the \(d(t)\) curve,

\[
d_{\text{contr}} = \frac{1}{m} \sum_{i}^{m} \max(d(t_k)) - \frac{1}{n} \sum_{j}^{n} \min(d(t_k)).
\]  

(2)

Average velocity defined for each timepoint (i.e. frame) using the mean displacements \(d(t_k)\),

\[
v(t_k) = \frac{\Delta d}{\Delta t} = \frac{d_{k+1} - d_{k-1}}{t_{k+1} - t_{k-1}}.
\]  

(3)

The maximal contraction and relaxation velocities then correspond to maxima and minima of the \(v(t_k)\) curve,

\[
v_{\text{max,contr}} = \frac{1}{m} \sum_{i}^{m} \max(v(t_k)) ; \quad v_{\text{max,relax}} = \frac{1}{n} \sum_{j}^{n} \min(v(t_k)).
\]  

(4)
To determine frequency of contraction, there are two possibilities.

(i) The signal can be Fourier transformed. Then the peaks correspond to main frequencies of the signal.

\[
\begin{align*}
\text{Figure 10: Fourier transformed signal}
\end{align*}
\]

(ii) Use the definition of frequency as inverse of the period \( T = \frac{1}{f} \). Using the displacement signal \( d(t_k) \), it is easy to draw periods on the curve. For multiple contractions, the mean can then be computed,

\[
\begin{align*}
f &= \left( \frac{1}{m} \sum_{i} T_i \right)^{-1} .
\end{align*}
\]

Method (ii) is probably the safer choice, as it is quite clear what is selected, especially in cases where the curve is not really periodic.

\[
\begin{align*}
\text{Figure 11: Displacement plot with time period}
\end{align*}
\]

Displays the mean sarcomere length in the cell body over time. The user has to select maxima and minima, in order to calculate the average sarcomere shortening,

\[
\begin{align*}
\text{shortening} &= \frac{1}{m} \sum_{i} \max (\ddot{s}(t_k)) - \frac{1}{n} \sum_{j} \min (\ddot{s}(t_k)) .
\end{align*}
\]

The mean over average sarcomere lengths in time is also shown.

Plot of the deviation of sarcomeres inside the cell as a function of time. No user input required.

It is difficult to evaluate the contraction time of one contraction, as it is hard to determine when it exactly starts and ends. However one can take a value that scales proportionally to contraction time: we take the
time between a velocity maximum and minimum of a contraction cycle,

\[ \hat{t} = \frac{1}{m} \sum_{i} \left\| \left[ t_{k|\max} v(t_k) \right] - \left[ t_{k|\min} v(t_k) \right] \right\|. \]  

\[ (7) \]

Figure 12: Velocity plot with \( \hat{t} \)

\[ \ell_5 \] Ratio of the cell that is taken by sarcomeres,

\[ \ell = \frac{A_{\text{cell}} \cap A_{\text{sarco}}}{A_{\text{cell}}} \]  

\[ (8) \]

\[ \ell_5 \] Deviation of myofibril orientation angle, as a measure of how organized the myofibrils are. No user input required.

\[ \ell_6 \] Displays the evolution of the mean of the standard deviation of the direction angles of displacements inside the cell (measure of spacial synchronicity). No user input required.

\[ \ell_7 \] Displays temporal synchronicty: heatplot/histogram of the synchronicity \( \delta \).

\[ \ell_8 \] Tag if there are double peaks.

\[ \ell_9 \] Tag if cell does not look useful for further analysis.

\[ \ell_{10} \] Go through all the videos and input parameters. Also gives a preview of the current cell outline or ROI.

\[ \ell_{11} \] Curve data and generated parameters are saved in the result file. The user can optionally select to also save curve plots. This then closes the window and concludes the analysis.

3 Folder & Files Structure

- Creation of a temporary folder for each video in the path of the Lifeact GUI:
Figure 13: Folder tree for temporary data

- Creation of a result folder for each video in the path of the video file:
Figure 14: Folder tree for result data

- For each cell video, a main Excel result file is created, which contains the microscope data, the curve data as well as the generated parameters. The file is organized into separate worksheets.

Figure 15: Excel result file
The following document is a tutorial on our TFM analysis tool. It serves as an introductory getting-started guide.

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1 Requirements and Comments

• The code has been tested on all versions of Matlab between R2014a and R2016a. It has been tested for Windows (7, 8, 10), OS X (10.9) and Linux (Arch). The GUI looks best on Windows, with minor scaling issues on the other platforms.

• The code uses some third-party packages, that we include in our repository. This is the complete list:
  
  - Ncorr\(^1\): DIC algorithms.
  - Bio-Formatstoolbox\(^2\): read image and video formats.
  - xwrite\(^3\): write results to Excel files.
  - peakdet\(^4\): determine peaks on noisy curves.
  - statusbar\(^5\) and enableDisableFig\(^6\): freeze the windows during calculations and estimate runtime.
  - freezeColors\(^7\) and COLORMAP and COLORBAR utilities\(^8\): display overlays on figures.
  - Exportfig\(^9\): flexible plot exports.

• On recent versions of Matlab, the GUI produces a variety of warnings related to packages used for blocking the interface during calculation. These can safely be ignored.

• During execution, temporary files as well as result data is written to the disk. Thus it is advisable to have at least a few hundred MB of free disk space. Also, all the functions and the file structure has to be left unaltered, and put in a place that has writing permissions.

• Windows will generally freeze during intense calculation to prevent all user input, then resume post-computationally. Calculations can however be stopped using the usual \text{ctrl} + \text{C} key combination. It is mandatory to close all windows with there respective \text{OK} buttons, not using the OS window close button.

\(^1\)http://ncorr.com/
\(^2\)http://www.openmicroscopy.org/site/support/bio-formats5/users/matlab/
\(^3\)http://www.mathworks.com/matlabcentral/fileexchange/38591-xlwrite--generate-xls-x--files-without-excel-on-mac-linux-win
\(^4\)http://www.billauer.co.il/peakdet.html
\(^5\)http://www.mathworks.com/matlabcentral/fileexchange/14773-statusbar
\(^6\)http://www.mathworks.com/matlabcentral/fileexchange/15895-enable-disable-figure
\(^7\)http://www.mathworks.com/matlabcentral/fileexchange/7943-freezecolors---unfreezecolors
\(^8\)http://www.mathworks.com/matlabcentral/fileexchange/24371-colormap-and-colorbar-utilities--jul-2014-
\(^9\)http://www.mathworks.com/matlabcentral/fileexchange/727-exportfig
2 Tutorial

The main window is opened by executing `tfm_gui_main.m`, and changing the working directory to the current folder. Figure (1) shows the main window of the analysis.

![Main window](image1)

**Figure 1:** Main window; in blue, the relevant sections of the tutorial

The analysis is split into four parts, each of them requiring user setup and review. The order of going through these is by increasing number.

Steps which are not accessible at user’s current stage are greyed out, steps which have been completed show up green.

2.1 Initialization Window

![Initialization window](image2)

**Figure 2:** Initialization window

One or more videos must be added to the analysis. Videos can be in the following formats:
• *.czi video files
• *.avi video files
• *.tiff image stacks

It is also possible to indicate a folder containing *.png images. Images are read in based on the order that they are listed in the folder, so images should be in the correct order, e.g. {im_001.png, im_002.png, im_003.png,...}. Be careful to pad numbers in file names with zeros, im_11.png generally list before im_2.png.

Once multiple videos are loaded, it is possible to review and browse through them. The axes displays the first frame of the respective video, and the video information is chosen accordingly.

Videos can be deleted from the analysis list, after they have been loaded. This may be useful if a whole list has not been input, but one video is not suitable for analysis.

Some information about the video is needed, in particular the framerate, the number of frames and the resolution. For indentification purposes in view of post-processing, cells should also be assigned a unique and characterizing name tag. No two videos are allowed the same cell name!

Depending on the format of the videos, the metadata is accessed automatically.

• For *.czi video files: whole metadata is available.
• For *.avi video files, *.tiff image stacks, image folders: user has to input framerate and resolution, whereas the number of frames is detected.

Sometimes there can be unwanted information in the videos, such as parts of a second cell. We can choose to draw a mask of the area that interests us. If a mask already exists from a previous analysis (such as a Brightfield or Lifeact analysis), it can be loaded.

Option to save the mask from the previous step. Note that there is no need to save for the program to remember the choice. It can however be useful to save in case the video is processed again. The video information is saved in the cell-specific Excel sheet. For more information about this, see section 3 on folder and files organization.

Based on video format, panels are enabled and disabled in a smart way. For instance for videos where the framerate is already available, no user input is required, which causes the respective input boxes to be greyed.

In case that the [OK] button is activated, but important information is still missing, e.g. the conversion factor for one video has not been entered, a reminder message will pop up.

Figure 3: Example of a masked image
2.2 Displacement Window

Displacements are computed with the Ncorr DIC code.

For DIC, the important parameters are subset size and radius. The size parameter is a tradeoff between accuracy and time-efficiency. Ideally it would be set very low (ideally 0) to get very high resolution, whereas large values produce an excessive smoothening effect. The radius is pretty tricky to set. It should ideally be as small as possible without getting noise effects.

To improve performance, only areas inside the cell outline are considered for displacements.

All videos are processed with identical analysis settings. The preview gives a heatmap preview of the current frame, with a fixed pre-set axis scale and vector length. For some videos, the preview thus might not look amazing, however usually this is a scaling issue.

Displacements are calculated with an arbitrary reference frame, the first frame of the video. For display and interpretation purposes, it is generally favourable to have a relaxed cell state as reference. This step smart-guesses the optimal reference frame.

The reference auto-guessing usually is very reliable. However there are cases, where the prediction is not accurate, e.g. when the videos are really small, or the cell movement is particularly unsynchronized, coupled with excessive image noise. A new reference value can be selected by picking a point as reference or inputting a frame number.

The contracted frame is not used for further calculations, so it is not necessary to change this, too. The reason the guess for the contracted frame is given is that for the unlikely case that the auto-guess failed, usually the issue can be fixed by exchanging relaxed and contracted guesses.

The previews should be visually inspected for all videos. Afterwards, displacement data has to transformed to the new reference. In addition, the synchronicity calculation is performed at this stage.
A lot of data is generated during the DIC analysis, which can optionally be stored for further processing. The options here are: saving the full displacement field in matrix form, as they are used during the analysis, and saving the preview heatmaps.

**Important Note:** Saving takes some time and should only be enabled if necessary.

The window is closed and the selected saving options are written to the disk. All Excel result files are updated with reference and contracted frame.

### 2.3 Traction Force Window

![Force window](tfm_gui_tfm.png)

**Figure 5:** Force window

- The regularization parameter is guessed in an automated process using the $L$-curve criterion.

- The stiffness properties for the used gel should be modified here. Also the bounds for the heatplots can be tuned. Change the limits, update, and observe the changes in the preview, which previews stresses between contracted and relaxed state (i.e. the maximal stresses).

- Calculate stress fields for all videos using the defined settings.

- Option to save the generated stress results and heatmaps.

- Saves the stress, forces and heatmap files (if desired) and closes the displacement window.
2.4 Parameter Window

![Parameter window](image)

**Figure 6: Parameter window**

**Note:** all of the following parameters can be left empty. If for instance one curve does not look good, and it is not possible to extract reliable information, just leave it out. The associated parameter will appear as NaN, which is left empty in Excel later.

- **a:** The maxima and minima of the displacement and velocity curves can be estimated in an automated way. This is not always accurate, and should be reviewed and corrected.

- **b:** Input the aspect ratio of patterns.

- **c:** Average displacement defined for each timepoint (i.e. frame) as the mean of all displacements inside cell blob,

\[
d(t_k) = \frac{1}{N} \sum_{k} \sqrt{u_{k,x}^2 + u_{k,y}^2}.
\]  

The contraction displacement then is the total distance between relaxed and contracted state, or in other words, the distance between minima and maxima on the \(d(t)\) curve,

\[
d_{\text{con}} = \frac{1}{m} \sum_{i} \max_{k} (d(t_{k})) - \frac{1}{n} \sum_{j} \min_{k} (d(t_{k})).
\]
Average velocity defined for each timepoint (i.e. frame) using the mean displacements \( d(t_k) \),

\[
v(t_k) = \frac{\Delta d}{\Delta t} = \frac{d_{k+1} - d_{k-1}}{t_{k+1} - t_{k-1}}
\]  

The maximal contraction and relaxation velocities then correspond to maxima and minima of the \( v(t_k) \) curve,

\[
v_{\text{max,contr}} = \frac{1}{m} \sum_{i=1}^{m} \max (v(t_k)) ; \quad v_{\text{max,relax}} = \frac{1}{n} \sum_{j=1}^{n} \min (v(t_k)) .
\]  

To determine frequency of contraction, there are two possibilities.

(i) The signal can be Fourier transformed. Then the peaks correspond to main frequencies of the signal.
(ii) Use the definition of frequency as inverse of the period $T = \frac{1}{f}$. Using the displacement signal $d(t_k)$, it is easy to draw periods on the curve. For multiple contractions, the mean can then be computed,

$$f = \left( \frac{1}{m} \sum_{i} T_i \right)^{-1}.$$  

\[(5)\]

Method (ii) is probably the safer choice, as it is quite clear what is selected, especially in cases where the curve is not really periodic.

Forces are transformed into local cell coordinates, to differentiate between major and minor cell axis components $F_x$ and $F_y$. Again, for the total generated force of a timepoint, the sum over all datapoints is taken.

$$F_x(t_k) = \sum_k F_{k,x} \quad , \quad F_y(t_k) = \sum_k F_{k,y} \quad , \quad F(t_k) = \sum_k \sqrt{F_{k,x}^2 + F_{k,y}^2}$$  

\[(6)\]

The generated force for contraction corresponds to the difference between relaxed and contracted force points on the $F_i$ curves, or in other words the difference between minima and maxima,

$$F_{\text{contr}} = \frac{1}{m} \sum_{i} \max(F(t_k)) - \frac{1}{n} \sum_{j} \min(F(t_k)).$$  

\[(7)\]

Total power is calculated by multiplying total force and velocity of each spacial point at a given time point.

$$P(t_k) = \sum_k F_k \cdot v_k$$  

\[(8)\]
The maximal contraction and relaxation power then correspond to maxima and minima of the $P(t_k)$ curve,

$$P_{\text{max,contr}} = \frac{1}{m} \sum_{i}^{m} \max_{i} (P(t_k)) ; \quad P_{\text{max,relax}} = \frac{1}{n} \sum_{j}^{n} \min_{j} (P(t_k)) .$$

(9)

It is difficult to evaluate the contraction time of one contraction, as it is hard to determine when it exactly starts and ends. However one can take a value that scales proportionally to contraction time: we take the time between a power maximum and minimum of a contraction cycle,

$$\hat{t} = \frac{1}{m} \sum_{i}^{m} \left\| t_k \max_{i} P(t_k) - t_k \min_{i} P(t_k) \right\|. $$

(10)

Figure 12: Power plot

Figure 13: Power plot with $\hat{t}$

- Tag if there are double peaks.
- Tag if cell does not look useful for further analysis.
- Go through all the videos and input parameters. Also gives a preview of the current cell outline or ROI.
- Curve data and generated parameters are saved in the result file. The user can optionally select to also save curve plots. This then closes the window and concludes the analysis.

August 28, 2016
3 Folder & Files Structure

• Creation of a temporary folder for each video in the path of the Lifeact GUI:

  Folder tree for temporary data

• Creation of a result folder for each video in the path of the video file:
Figure 15: Folder tree for result data

- For each cell video, a main Excel result file is created, which contains the microscope data, the curve data as well as the generated parameters. The file is organized into separate worksheets.