

1 **Versatile open software to quantify cardiomyocyte and cardiac**  
2 **muscle contraction *in vitro* and *in vivo***

3

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## 26 **Abstract**

27 Contraction of muscle reflects its physiological state. Methods to quantify contraction are often  
28 complex, expensive and tailored to specific models or recording conditions, or require specialist  
29 knowledge for data extraction. Here we describe an automated, open-source software tool  
30 (MUSCLEMOTION) adaptable for use with standard laboratory and clinical imaging equipment that  
31 enables quantitative analysis of normal cardiac contraction, disease phenotypes and pharmacological  
32 responses. MUSCLEMOTION allowed rapid and easy measurement of contractility in (i) single  
33 cardiomyocytes from primary adult heart and human pluripotent stem cells, (ii) multicellular 2D-  
34 cardiomyocyte cultures, 3D engineered heart tissues and cardiac organoids/microtissues *in vitro* and  
35 (iii) intact hearts of zebrafish and humans *in vivo*. Good correlation was found with conventional  
36 measures of contraction in each system. Thus, using a single method for processing video recordings,  
37 we obtained reliable pharmacological data and measures of cardiac disease phenotype in experimental  
38 cell- and animal models and human echocardiograms.

39

## 40 **Introduction**

41 The salient feature of cardiomyocytes (CMs) is their ability to undergo cyclic contraction and  
42 relaxation, a feature critical for cardiac function. In many research laboratories and clinical settings it  
43 is therefore essential that cardiac contraction can be quantified at multiple levels, from single cells to  
44 multicellular or intact cardiac tissues. Measurement of contractility is relevant for analysis of disease  
45 phenotypes, cardiac safety pharmacology, and longitudinal measures of cardiac function over time,  
46 both *in vitro* and *in vivo*. In addition, human genotype-phenotype correlations, investigation of cardiac  
47 disease mechanisms and the assessment of cardiotoxicity are increasingly performed on human  
48 induced pluripotent stem cells (hiPSCs) derived from patients<sup>1-3</sup>. Many of these studies are carried out  
49 in non-specialist laboratories so that it is important that analysis methods are simplified such that they  
50 can be used anywhere with access to just standard imaging equipment. Here, we describe a single  
51 method with high versatility that can be applied to most imaging outputs of cardiac contraction likely  
52 to be encountered in the laboratory or clinic.

53 Electrical and calcium signals are usually quantified *in vitro* using established technologies such as  
54 patch clamp electrophysiology, multi electrode arrays, cation-sensitive dyes or cation-sensitive genetic  
55 reporters<sup>4</sup>. Although experimental details differ among laboratories, the values for these parameters  
56 are with some approximations comparable across laboratories, cardiomyocyte source and cell culture  
57 configuration (e.g. single cells, multicellular 2-Dimensional (2D) CM monolayers, 3-Dimensional  
58 (3D) cultures)<sup>5,6</sup>. However, there is no comparable method for measuring cardiac contraction across  
59 multiple platforms, despite this being a crucial functional parameter affected by many diseases or  
60 drugs<sup>7</sup>. We have developed a method to address this that is built on existing algorithms and is fully  
61 automated, but most importantly can be used on videos, image stacks or image sequences loaded in the  
62 open source image processing program ImageJ<sup>8</sup>. Moreover, it is an open source, dynamic platform that  
63 can be expanded, improved and integrated for customized applications. The method, called  
64 MUSCLEMOTION, determines dynamic changes in pixel intensity between image frames and  
65 expresses the output as a relative measure of displacement during muscle contraction and relaxation.  
66 We applied the concept to a range of biomedical- and pharmacologically relevant experimental models  
67 that included single hPSC-CMs, patterned- or 2D cultures of hPSC-CMs, cardiac organoids,  
68 engineered heart tissues (EHTs) and isolated adult rabbit CMs. Results were validated by comparing  
69 outputs of the tool with those from three established methods for measuring contraction: optical flow,  
70 post deflection and fractional shortening of sarcomere length. These methods have been tailored to (or  
71 only work on) specific cell configurations. Traction force microscopy, fractional shortening of  
72 sarcomere length and microposts are predominantly suitable for single cells<sup>8,9</sup>. Cardiomyocyte edge or  
73 perimeter detection is suitable for adult CMs but challenging for immature hPSC-CMs due to poorly  
74 defined plasma membrane borders and concentric contraction<sup>10</sup>, while large post deflection is suitable  
75 for EHTs or small cardiac bundles<sup>11</sup> but less so for single cells. Our MUSCLEMOTION software by  
76 contrast can be used for all of these applications without significant adaptations. Furthermore, it can be  
77 used for multi-parameter recording conditions and experimental settings using transmitted light  
78 microscopy, fluorescent membrane labeling, fluorescent beads embedded in soft substrates or patch  
79 clamp video recordings. Drug responses to positive and negative inotropic agents were evaluated  
80 across four different laboratories in multiple cell configurations using MUSCLEMOTION with

81 reliable predictions of drug effects from all laboratories. Furthermore, MUSCLEMOTION was also  
82 applicable to optical recordings of zebrafish hearts *in vivo*, where it represented a significant time-  
83 saving in analysis, and in human echocardiograms. This versatile tool thus provides a rapid and  
84 straightforward way to detect disease phenotypes and pharmacological responses *in vitro* and *in vivo*.

85

## 86 **Methods**

87 Extended methods are in the Supplementary Information. The datasets generated and/or analyzed  
88 during the current study are available from the corresponding authors on reasonable request.

### 89 **Code Availability**

90 MUSCLEMOTION source code is included in the Supplementary Material and is available for use  
91 and further development.

### 92 **Model Cell**

93 The *in silico* cardiomyocyte-like model (**Fig. 1d,f,g**) was created using Blender v2.77.

### 94 **Optical Flow analysis**

95 Optical flow analysis was implemented in LabVIEW as described by Hayakawa et al.<sup>12,13</sup>.

### 96 **hPSC Culture and Differentiation**

97 hPSCs from multiple independent cell lines (**Table S1**) were differentiated to CMs as previously  
98 described<sup>14-17</sup>, or with the Pluricyte® Cardiomyocyte Differentiation Kit (Pluriomics b.v.) according to  
99 the manufacturer's protocol. Experiments were performed at 18-30 days after initiation of  
100 differentiation, depending on the cell source and configuration. Pluricytes® were kindly provided by  
101 Pluriomics b.v.

### 102 **Patch Clamp Recordings on hPSC-CMs**

103 Electrophysiological recordings of isolated hPSC-CMs were performed as previously described<sup>16</sup>.

### 104 **Movement of embedded beads**

105 Gelatin-patterned polyacrylamide gels containing fluorescent beads were generated and analyzed as  
106 described previously<sup>18</sup>.

107

108 **Monolayers of hPSC-CMs**

109 25k-40k cells were plated per Matrigel-coated glass ø10 mm coverslip.

110 **Cardiac Organoids**

111 Cardiac organoids composed of hPSC-CMs and hPSC-derived endothelial cells, were generated as  
112 previously described<sup>17</sup>.

113 **Adult cardiomyocytes**

114 CMs were isolated from New Zealand White male rabbits as previously described<sup>19</sup>.

115 **Membrane labelling**

116 hPSC-CMs were plated on Matrigel-coated glass-bottom 24-well plates and labelled with CellMask  
117 Deep Red according to the manufacturer's instructions.

118 **Engineered heart tissues**

119 EHTs were generated and analyzed as previously described<sup>14</sup>.

120 **Zebrafish hearts**

121 Zebrafishes hearts were recorded, treated and analysed as previously described<sup>23</sup>.

122 **Echocardiograms**

123 Anonymized ultrasounds of 5 adult patients were selected from the echocardiography database of the  
124 Leiden University Medical Center.

125 **Statistics**

126 One-way ANOVA for paired or unpaired measurements was applied to test the differences in means  
127 on normalized drug effects. P-values obtained from two-tailed pairwise comparisons were corrected  
128 for multiple testing using Bonferroni's method. Statistical analyses were performed with R v3.3.3. P-  
129 values lower than 0.05 were considered statistically significant and indicated with an asterisk (\*).

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## 134 Results

### 135 Algorithm development

136 The principle underlying the algorithm of MUSCLEMOTION is the assessment of contraction using  
137 an intuitive approach quantifying absolute changes in pixel intensity between a reference frame and  
138 the frame of interest, which can be described as

139

$$140 \quad |img_i - img_{ref}| = img_{result}$$

141

142 where  $img_i$  is the frame of interest,  $img_{ref}$  is the reference frame and  $img_{result}$  is the resulting  
143 image. For every pixel in the frame, each reference pixel is subtracted from the corresponding pixel of  
144 interest and the difference is presented in absolute numbers. Unchanged pixels result in low (black)  
145 values, while pixels that are highly changed result in high (white) values (**Fig. 1a**). Next, the mean  
146 pixel intensity of the resulting image is measured. This is a quantitative measure of how much the  
147 pixels have moved compared to the reference frame: more white pixels indicate more changing pixels  
148 and, thus, more displacement. When a series of images is analysed relative to the same reference  
149 image, the output describes the accumulated displacement over time (measure of displacement, **Fig.**  
150 **1b**).

151 However, if a series of images is analysed with a reference frame that depends on the frame of interest  
152 (e.g.  $img_{ref} = img_{i-1}$ ), this results in a measure of the relative displacement per interframe interval.

153 We defined this parameter as contraction velocity (measure of velocity, **Fig. 1b**).

154 Since velocity is the first derivative of displacement in time, the first derivative of the measure  
155 of displacement should resemble the measure of velocity derived from image calculations. To test the  
156 linearity of the method, three movies of moving blocks were analysed. The block moved back and  
157 forth at two different speeds in each direction (where  $v_2 = 2 \cdot v_1$ ): i) along the x-axis, ii) along the y-  
158 axis and iii) along both axes (**Movie S1**). As expected, the measure of displacement and velocity  
159 showed a linear correlation (**Fig. S1**). This does not hold when the position of the block in  $img_i$  does  
160 not overlap the position of the block in  $img_{ref}$ , with a consequent saturation in the measure of

161 displacement (i.e. max pixel white value, **Fig. S2**). Therefore, comparison of the differentially derived  
162 velocities should approximately overlap in the absence of pixel saturation. This was used as a  
163 qualitative parameter to determine whether the algorithm outputs were reliable.

164

### 165 **Algorithm implementation**

166 MUSCLEMOTION was then modified to handle typical experimental recordings by (i) improving the  
167 signal-to-noise ratio (SNR), (ii) automating reference frame selection and (iii) programming built-in  
168 checks to validate the generated output data (**Fig. 1c**). The SNR was increased by isolating the pixels  
169 of interest in a three-step process: i) maximum projection of pixel intensity in the complete  
170 displacement stack, ii) creation of a binary image of this maximum projection with a threshold level  
171 equal to the mean grey value plus standard deviation and iii) multiplication of the pixel values in this  
172 image by the original displacement and speed of the displacement image stack (**Fig. S3**). This process  
173 allowed the algorithm to work on a region of interest with movement above the noise level only.

174 Next, a method was developed to identify the correct  $img_{ref}$  from the speed of displacement image  
175 stack by comparing values obtained from the frame-to-frame calculation with their direct neighbouring  
176 values, while also checking for the lowest absolute value (**Fig. S4**).

177 The reliability of MUSCLEMOTION for structures with complex movements was validated using a  
178 custom-made contracting 3D “synthetic CM” model (**Fig. 1d,f,g**) that was adapted to produce  
179 contractions with known amplitude and duration. Linearity was preserved during the analysis of the  
180 contraction and velocity; other output parameters of the analysis matched the input parameters (**Fig.**  
181 **1e**). A second 3D model (**Fig. 1g**), with a repetitive pattern aimed to create out-of-bounds problems  
182 was also generated. As expected, contraction amplitude information here was not linear (**Fig. 1e**),  
183 although contraction velocity and temporal parameters did remain linear (**Fig. 1e,g**). To mitigate this  
184 problem, we implemented an option for a 10-sigma Gaussian blur filter that can be applied on demand  
185 to biological samples that presented highly repetitive patterns (e.g. sarcomeres in adult CMs).

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188 **Algorithm application to multiple cell configurations and correlation with existing gold**  
189 **standards**

190 This set of experiments aimed to investigate the versatility of MUSCLEMOTION and examine how  
191 its performance compared with standard measures used in each system: i) optical flow for isolated  
192 hPSC-CMs, monolayers and organoids; ii) post deflection for EHT; iii) sarcomere length fractional  
193 shortening for adult CMs. Remarkably, standard methods currently used measure only contraction or  
194 contraction velocity. Linearity was preserved in all cases during the analyses, demonstrating the  
195 reliability of the results (**Fig. S5**).

196 First, single hPSC-CMs (**Fig. 2a, Movie S2**) exhibited concentric contraction (**Fig. 2a ii**) and  
197 contraction velocity amplitudes correlated well with the amplitudes obtained by optical flow analysis  
198 ( $R^2 = 0.916$ ) (**Fig. 2a v**). In contrast to single cells, the area of displacement for hPSC-CM monolayers  
199 was distributed heterogeneously throughout the whole field (**Fig. 2b ii, Movie S3**). Optical flow  
200 analysis was compared with our measure of velocity (**Fig. 2b iv**); this showed a good linear correlation  
201 ( $R^2 = 0.803$ ) (**Fig. 2b v**). Complex (mixed, multicellular) 3D configurations were also investigated by  
202 analyzing hPSC-derived cardiac organoids<sup>17</sup> (**Movie S4**) and EHTs<sup>14</sup> (**Movie S5**). Cardiac organoids  
203 showed moderate levels of displacement throughout the tissue (**Fig. 2c ii**), while the EHTs showed  
204 high deflection throughout the bundle (**Fig. 2d ii**). The contraction velocity of the organoids correlated  
205 well with the output of optical flow analysis ( $R^2 = 0.747$ , **Fig. 2c v**). Similarly, contraction amplitudes  
206 in EHTs showed high linear correlation ( $R^2 = 0.819$ ) with the absolute force values derived from  
207 measurement of pole deflection (**Fig. 2d v**). Finally, single adult rabbit ventricular CMs were analyzed  
208 (**Fig. 2e, Movie S6**). Large displacements were evident around the long edges of the CM (**Fig. 2e ii**).  
209 These cells were analyzed with a 10-sigma Gaussian blur filter, which also minimized (unwanted)  
210 effects of transverse movements on contraction patterns. Linearity was preserved (**Fig. S5**) despite the  
211 repetitive pattern of the sarcomeres and this resulted in accurate measures of both contraction (**Fig. 2e**  
212 **iii**) and speed of contraction (**Fig. 2e iv**). The contraction amplitude of the adult CMs stimulated at 1  
213 Hz correlated well with the output of sarcomeric shortening using fast Fourier transform analysis<sup>20</sup> ( $R^2$   
214  $= 0.871$ , **Fig. 2e v**). Thus, the MUSCLEMOTION algorithm yielded data in these initial studies  
215 comparable with methods of analysis tailored for the individual platforms.

## 216 **Application of MUSCLEMOTION to multiple imaging and recording platforms**

217 To examine whether MUSCLEMOTION could potentially be used in applications that measure other  
218 aspects of CMs functionality in parallel, we first determined the electrophysiological properties of  
219 hPSC-CMs using patch clamp whilst recording their contractile properties through video imaging.  
220 This allowed simultaneous quantitative measurement of action potentials (APs) and contraction (**Fig.**  
221 **3a**), for in-depth investigation of their interdependence. We observed a typical<sup>21</sup> profile of AP  
222 followed by its delayed contraction.

223 To measure contractile force in combination with contractile velocity in single CMs, we integrated  
224 fluorescent beads into polyacrylamide substrates patterned with gelatin (**Fig. 3b**), where the  
225 displacement of the beads is a measure of CM contractile force<sup>18</sup> (**Movie S7**).

226 Similarly, effective quantification of contraction profiles was obtained for fluorescently labeled hPSC-  
227 CM monolayer cultures (**Fig. 3c, Movie S8**), allowing MUSCLEMOTION to be integrated on high  
228 speed fluorescent microscope systems for automated data analysis.

229

## 230 **Application of MUSCLEMOTION to drug responses in different cell models in different** 231 **laboratories**

232 Having shown that MUSCLEMOTION was fit-for-purpose in analyzing contraction over a variety of  
233 platforms, we next sought to demonstrate its ability to detect the effects of positive and negative  
234 inotropes. This is essential for ensuring the scalability of the tool over multiple platforms, particularly  
235 in the context of hiPSC-CMs where regulatory authorities and pharmaceutical companies are  
236 interested in using these cells as human heart models for drug discovery, target validation or safety  
237 pharmacology<sup>22</sup>. For isoprenaline (ISO) and nifedipine (NIFE) the main parameters of interest are:  
238 contraction amplitude (ISO, NIFE), relaxation time (ISO) and contraction duration (NIFE).

239

240 The relaxation time of spontaneously beating isolated hPSC-CMs on gelatin patterned polyacrylamide  
241 substrates treated with ISO significantly decreased as expected at doses higher than 1 nM. Similar to  
242 what has been reported<sup>27</sup>, contraction amplitude decreased at doses higher than 1 nM. NIFE treatment  
243 decreased both contraction amplitude and duration starting from 3 nM, respectively (**Fig. 4a**). In paced

244 (1.5 Hz) hPSC-CMs monolayers, no significant effects were measured after addition of ISO on either  
245 relaxation time or contraction amplitude. NIFE caused a progressive decrease in contraction duration  
246 and amplitude in a concentration-dependent manner starting at 100 nM (**Fig. 4b**). Similarly, cardiac  
247 organoids paced at 1.5 Hz showed no significant effects on both relaxation time and contraction  
248 amplitude with ISO, while both parameters decreased after NIFE, starting from 100 nM and 300 nM,  
249 respectively (**Fig. 4c**). EHTs paced at 1.5 times baseline frequency and analyzed with  
250 MUSCLEMOTION showed a positive inotropic effect starting from 1 nM ISO and a negative  
251 inotropic effect starting at 30 nM NIFE as previously reported<sup>14</sup> (**Fig. 4d**).  
252 Paced (1 Hz) adult rabbit CMs exhibited no significant increase in relaxation time and contraction  
253 amplitude at any ISO concentration. At concentrations higher than 3 nM, adult CMs exhibited after-  
254 contractions and triggered activity during diastole, which hampered their ability to be paced at a fixed  
255 frequency. No significant effects were observed on contraction duration with NIFE, while contraction  
256 amplitude significantly decreased in a dose-dependent manner starting from 100 nM (**Fig. 4e**). Data  
257 generated by post deflection and sarcomere fractional shortening are available for comparison  
258 purposes in **Fig. S6**.

259

### 260 **Analysis of disease phenotypes in vivo**

261 To extend analysis to hearts *in vivo*, we took advantage of the transparency of zebrafish, which allows  
262 recording of contracting cardiac tissue *in vivo* (**Fig. 5a, Movie S9**). It was previously shown that  
263 mutations in G protein  $\beta$  subunit 5 (*GNB5*) are associated with a multisystem syndrome in human,  
264 with severe bradycardia at rest. Zebrafish with loss of function mutations in *gnb5a* and *gnb5b* were  
265 generated. Consistent with the syndrome manifestation in patients, zebrafish *gnb5a/gnb5b* double  
266 mutant embryos showed severe bradycardia in response to parasympathetic activation<sup>23</sup>. Irregularities  
267 in heart rate were visually evident and were clearly distinguishable from the wild type counterpart  
268 after analysis with MUSCLEMOTION (**Fig. 5b**). Quantification of the heart rate of these zebrafishes  
269 with MUSCLEMOTION highly correlated ( $R^2 = 0.98$ ) with the results of the published manual  
270 analyses<sup>23</sup> (**Fig. 5c**). There was however, a striking time-saving for operators in carrying out the  
271 analysis using the algorithm (5-10 times faster than manual analysis; 150 recordings were analysed in

272 5 hours versus 4 days) without compromising accuracy of the outcome. Qualitative analysis of  
273 contraction patterns allowed rapid discrimination between arrhythmic vs non-arrhythmic responses to  
274 carbachol treatment (**Fig. 5c**).

275 Finally, we examined human echocardiograms from five healthy and cardiomyopathic individuals  
276 (**Fig. 5d**). To assess ventricular function, videos were cropped to exclude movement contributions of  
277 the atria and valves. MUSCLEMOTION enabled rapid quantification of temporal parameters from  
278 standard ultrasound echography (**Fig. 5e**) such as time-to-peak, relaxation time, RR interval and the  
279 contraction duration (**Fig. 5f**).

280

## 281 **Discussion**

282 A reliable and easy-to-use method to quantify cardiac muscle contraction would be of significant  
283 benefit to many basic and clinical science laboratories to characterize cardiac disease phenotypes,  
284 understand underlying disease mechanisms and predicting cardiotoxic effects of drugs<sup>14,24</sup>.  
285 Quantification of frame-to-frame differences in pixel intensity has been used in recent reports with  
286 success<sup>10</sup>; however, the full spectrum of applications for which these algorithms are relevant, how their  
287 output data correlates with gold standards in each system and software performance, specifications,  
288 license and software availability, have remained unclear.

289 Here we developed and tested a user-friendly, inexpensive, open source software platform that serves  
290 this purpose in a variety of biological systems of heart tissue. Its integration into current research  
291 practices would benefit data sharing, reproducibility, comparison and translation in many clinically  
292 relevant contexts<sup>25</sup>.

293 The linearity and reliability of MUSCLEMOTION were validated using a 3D reconstructed artificial  
294 CM which gave the expected linear correlations between known inputs and the outputs (**Fig. 1d-f**).  
295 When random repetitive patterns were applied, amplitude outputs differed from inputs, suggesting a  
296 potential limitation to measuring contraction amplitudes in highly repetitive biological samples (such  
297 as when sarcomere patterns are well-organized), while temporal parameters remained valid (**Fig.**  
298 **1d,e,g**). However, conditions such as these would be unlikely in standard biological samples, where

299 camera noise significantly reduces the possibility of saturating pixel movement. We partially  
300 attenuated this problem by applying, on user demand, a 10-sigma Gaussian blur filter which  
301 significantly increased the accuracy of MUSCLEMOTION with highly repetitive structures. Also, to  
302 increase reliability, we built in additional controls to detect any mismatches and errors.  
303 MUSCLEMOTION can automatically identify and select the reference frame and increase the signal-  
304 to-noise-ratio, features which were particularly relevant in reducing user bias and interaction while  
305 improving user experience. MUSCLEMOTION is valid in a wide range of illumination conditions  
306 without changing temporal parameters; however, exposure time was linearly correlated with  
307 contraction amplitude (**Fig. S7**). Batch mode analyses and data storage in custom folders were also  
308 incorporated to support overnight automated analyses. For accurate quantification of amplitude, time-  
309 to-peak and relaxation time, an appropriate sampling rate should be chosen. For applications similar to  
310 those described here, we recommend recording rates higher than 70 frames per second to sample  
311 correctly the fast upstroke of the time-to-peak typical of cardiac tissue. This recording rate is easily  
312 achievable even using smartphone slow motion video options (~120/240 frames per second), obviating  
313 the need for dedicated cameras and recording equipment if necessary.

314 We demonstrated excellent linear correlations between our software tool and multiple other standard  
315 methods independent of substrate, cell configuration and technology platform and showed that  
316 MUSCLEMOTION is able to capture contraction in a wide range of *in vivo* and *in vitro* applications  
317 (**Fig. 2** and **Fig. 3**). Specifically, we identified several advantages compared to optical flow algorithms  
318 in terms of speed and the absence of arbitrary binning factors or thresholds which, when modified,  
319 profoundly affect the results. One limitation compared to optical flow or EHT standard algorithm is  
320 that the tool lacks qualitative vector orientation, making it more difficult to assess contraction  
321 direction. Particularly important was the correlation with force data calculated from the displacement  
322 of flexible posts by EHTs. This indicates that when the mechanical properties of substrates are  
323 known<sup>26</sup>, MUSCLEMOTION allows absolute quantification of contractile force. Technical limitations  
324 of the EHT recording system allowed us to analyze only movies with JPEG compression; this resulted  
325 in loss of pixel information that might have negatively influenced the correlation shown. For better  
326 and more accurate results on contraction quantification, non-lossy/uncompressed video formats should

327 be used for recordings since individual pixel information is lost upon compression and therefore not  
328 available for analysis by MUSCLEMOTION.

329 We proposed and validated practical application in pharmacological challenges using multiple  
330 biological preparations recorded in different laboratories; this means that immediate use in multiple  
331 independent high-throughput drug-screening pipelines is possible without further software  
332 development being required, as recently applied for a drug screening protocol on cardiac organoids  
333 from hPSCs<sup>17</sup>. Intuitively, the possibility of having inter-assay comparisons will also be of particular  
334 relevance where comparisons of contraction data across multiple platforms are required by regulatory  
335 agencies or consortia (e.g. CiPA, CSAHi)<sup>5,6,22,27</sup>. Moreover, this might offer a quantitative approach to  
336 investigating how genetic or acquired diseases of the heart (e.g. cardiomyopathies<sup>7</sup>, Long QT  
337 Syndrome<sup>28</sup>), heart failure resulting from anticancer treatments<sup>29,30</sup> or maturation strategies<sup>18,31,32</sup> affect  
338 cardiac contraction. The possibility of linking *in vitro* with *in vivo* assays, with low cost technologies  
339 applicable with existing hardware certainly represents an advantage as demonstrated by automatic  
340 quantification of zebrafish heartbeats and human echocardiograms (**Fig. 5**). Overall, these results  
341 clearly demonstrated that contraction profiles could be derived and quantified in a wide variety of  
342 commonly used experimental and clinical settings. MUSCLEMOTION might represent a starting  
343 point for a swift screening method to provide clinically relevant insights into regions of limited  
344 contractility in the hearts of patients. We encourage further development of this open source platform  
345 to fit specific needs; future areas of application could include skeletal or smooth muscle in the same  
346 range of formats described here.

347 MUSCLEMOTION allows the use of a single, transparent method of analysis of cardiac contraction in  
348 many modalities for rapid and reliable identification of disease phenotypes, potential cardiotoxic  
349 effects in drug screening pipelines and translational comparison of contractile behaviour.

350

## 351 **Limitations**

352 Saturation of pixel movements may affect contraction amplitudes. However, as demonstrated with the  
353 artificial CM, contraction velocity and all temporal parameters remained valid. We also minimized the

354 impact of highly repetitive structures on the output of MUSCLEMOTION by applying a Gaussian  
355 filter, which also helped in reducing the impact of transverse movements on contraction profiles. High  
356 frequency contraction might complicate baseline detection, especially if the duration of the contracted  
357 state is similar to that of the relaxed (e.g. approaching sinusoidal). We have implemented a “*fast*  
358 *mode*” option that captures reliable baseline values even at high contraction rates. Furthermore,  
359 recordings must be free of moving objects (e.g. debris moved by flow, air bubbles) other than those of  
360 interest.

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371

## 372 **Conflict of interests**

373 MCL and PR are co-founders of Pluriomics B.V.

374 SGL and BF are co-founders of Clyde Biosciences Ltd.

375 ET, HA and MI are co-founders of EHT Technologies GmbH

376

## 377 **Author Contributions**

378 **SL:** project design, patch clamp, monolayer and organoids experiments, algorithm design, data  
379 analysis, statistics, wrote the manuscript.

380 **MBJ:** project design, monolayer, organoids and membrane labelling experiments, algorithm design,  
381 data analysis, statistics, wrote the manuscript.

382 **TLGJ:** project supervision, algorithm design, optical flow analyses.

383 **BJ:** supervision of zebrafish experiments.

384 **BM:** supervision of experiments on isolated hPSC-CM, cardiac organoids and monolayers.

385 **DRP:** supervision of cell culture for membrane labelling experiments.

- 386 **DC:** expert advice coordination of multi-center drug experiments under Crack-IT InPulse.
- 387 **DMAE:** designed and rendered the 3D artificial cells.
- 388 **ET:** supervision of experiments on Engineered Heart Tissues.
- 389 **GE:** generation of cardiac organoids and cell culture.
- 390 **CG:** cell culture for membrane labelling experiments.
- 391 **HA:** supervision on experiments on engineered heart tissues.
- 392 **HER:** advices and supervision on echocardiography data.
- 393 **JMRM:** echocardiography recordings and supervision on echocardiography data.
- 394 **KSM:** recordings and data analysis of zebrafish hearts.
- 395 **KCD:** recordings and data analysis of zebrafish hearts.
- 396 **LQ:** recordings and data analysis of adult rabbit cardiomyocytes.
- 397 **MI:** experiments and recordings of engineered heart tissues.
- 398 **MMPH:** cell culture for membrane labelling experiments.
- 399 **OVV:** supervision of experiments on cardiac organoids.
- 400 **PR:** supervision of drug tests experiments on aligned cardiomyocytes.
- 401 **RMC:** experiments on aligned cardiomyocytes.
- 402 **SU:** data analysis of engineered heart tissues.
- 403 **SGL:** project supervision and discussion.
- 404 **MCL:** project supervision and discussion, wrote the manuscript.
- 405 **BFL:** project supervision, algorithm design, discussion.
- 406

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488

489

## 490 **Figure Legends**

### 491 **Figure 1**

#### 492 **Algorithm construction and validation.**

493 **a)** Principle of pixel intensity difference by subtraction of  $img_{ref}$  of  $img_i$  and measurement of the non-  
494 zero area after image subtraction.

495 **b)** Principle of using pixel intensity difference as a measure of displacement and as a measure of  
496 velocity.

497 **c)** Schematic overview of MUSCLEMOTION. Green blocks indicate basic steps of the algorithm.  
498 Dark green blocks indicate important user input choices. Plots within light green blocks indicate  
499 results. Optional steps are shown in blue blocks, with graphical representation of the analysed  
500 parameters indicated by red lines. Three result files are generated containing the raw data:  
501 “contraction.txt”, “speed-of-contraction.txt” and “overview-results.txt”. Furthermore, three images  
502 showing relevant traces and a log file are generated and saved (not shown in schematic).

503 **d)** Schematic of the contractile pattern of the artificial cell and relative parameters corresponding to  
504 amplitude of contraction ( $A$ ), time-to-peak ( $t_1$ ) and relaxation time ( $t_2$ ).

505 **e)** Correlation between input ( $x$  axis) and output ( $y$  axis) parameters used to validate  
506 MUSCLEMOTION with two artificial cells.

507 **f-g)** Frame representing the two artificial cells built for MUSCLEMOTION validation and their  
508 relative output parameters.

509

### 510 **Figure 2**

#### 511 **Correlation of results with gold standards.**

512 **a)** Brightfield image of isolated hPSC-CMs (**i**), with maximum projection step visually enhanced with  
513 a fire Look Up Table (**ii**), contraction (**iii**) and velocity (**iv**) profiles of each individual beat have been  
514 generated by MUSCLEMOTION and temporally aligned; linear regression analysis between  
515 MUSCLEMOTION results ( $x$ -axis) and optical flow results ( $y$ -axis) (**v**).

516 **b)** Phase contrast image of hPSC-CM monolayers **(i)**, with maximum projection step visually  
517 enhanced with a fire Look Up Table **(ii)**, contraction **(iii)** and velocity **(iv)** profiles of each individual  
518 beat have been generated by MUSCLEMOTION and temporally aligned; linear regression analysis  
519 between MUSCLEMOTION results (x-axis) and those obtained with optical flow results (y-axis) **(v)**.  
520 **c)** Phase contrast image of cardiac organoids **(i)**, with maximum projection step visually enhanced  
521 with a fire Look Up Table **(ii)**, contraction **(iii)** and velocity **(iv)** profiles of each individual beat have  
522 been generated by MUSCLEMOTION and temporally aligned; linear regression analysis between  
523 MUSCLEMOTION results (x-axis) and those obtained with optical flow results (y-axis) **(v)**.

524 **d)** Live view of an EHT during contraction analysis. Scale bar = 1 mm. **(i)**, with maximum projection  
525 step visually enhanced with a fire Look Up Table **(ii)**, contraction **(iii)** and velocity **(iv)** profiles of  
526 each individual beat have been generated by MUSCLEMOTION and temporally aligned; linear  
527 regression analysis between MUSCLEMOTION results (x-axis) and those obtained with post  
528 deflection (y-axis) **(v)**.

529 **e)** Brightfield image of adult rabbit CMs **(i)**, with maximum projection step visually enhanced with a  
530 fire Look Up Table **(ii)**; contraction **(iii)** and velocity **(iv)** profiles of each individual beat have been  
531 generated by MUSCLEMOTION and temporally aligned; linear regression analysis between  
532 MUSCLEMOTION results (x-axis) and those obtained from sarcomere fractional shortening  
533 calculation with Fast Fourier Transform (y-axis) **(v)**.

534 For details on cell sources and cell lines please refer to the Supplementary Table 1.

535

536

### 537 **Figure 3**

#### 538 **Application of contraction tool to multiple biological situations.**

539 Representative examples with enhancement of moving pixels **(top)** and profiles **(bottom)** of  
540 contraction **(a-c, red)**, velocity **(a-c, black)** and voltage **(a, blue)** respectively obtained from high  
541 speed movies of patched hPSC-CMs **(a)**, aligned hPSC-CMs on polyacrylamide gels with fluorescent  
542 beads **(b)** and hPSC-CMs whose membranes have been labelled with CellMask Deep Red **(c)**.

543 For details on cell sources and cell lines please refer to the Supplementary Table 1.

544 **Figure 4**

545 **Pharmacological challenge with positive and negative inotropic compounds.**

546 **a)** Average dose-response curves (**black traces**) and single measurements for several parameters  
547 obtained in isolated, spontaneously beating, aligned hPSC-CMs treated with isoprenaline (**left, red**)  
548 and nifedipine (**right, green**).

549 **b)** Average dose-response curves (**black traces**) and single measurements for several parameters  
550 obtained from monolayers of hPSC-CMs treated with isoprenaline (**left, red**) and nifedipine (**right,**  
551 **green**).

552 **c)** Average dose-response curves (**black traces**) and single measurements for several parameters  
553 obtained in cardiac organoids treated with isoprenaline (**left, red**) and nifedipine (**right, green**).

554 **d)** Average dose-response curves (**black traces**) and single measurements for several parameters  
555 obtained in EHTs treated with isoprenaline (**left, red**) and nifedipine (**right, green**).

556 **e)** Average dose-response curves (**black traces**) and single measurements for several parameters  
557 obtained in adult rabbit CMs treated with isoprenaline (**left, red**) and verapamil (**right, green**).

558 Average data points (**black**) represent mean  $\pm$  standard error of mean. For details on cell sources and  
559 cell lines please refer to the Supplementary Table 1.

560 Data information: P-values DMSO versus dose. Panel a i) **0.3 nM**: 0.2897; **1 nM**:  $3.4 \cdot 10^{-6}$ ; **3 nM**:  
561  $3.8 \cdot 10^{-8}$ ; **10 nM**:  $7 \cdot 10^{-11}$ ; **30 nM**:  $7.3 \cdot 10^{-10}$ ; **100 nM**:  $2.4 \cdot 10^{-10}$ . Panel a ii) **0.3 nM**: 1; **1 nM**: 0.0645; **3**  
562 **nM**: 0.0136; **10 nM**:  $8.2 \cdot 10^{-5}$ ; **30 nM**: 0.0063; **100 nM**:  $2.4 \cdot 10^{-6}$ . (N=14; 14; 14; 14; 14; 14; 14)

563 Panel a iii) **3 nM**: 0.6533; **10 nM**:  $4 \cdot 10^{-5}$ ; **30 nM**:  $2 \cdot 10^{-9}$ ; **100 nM**:  $1.5 \cdot 10^{-15}$ . Panel a iv) **3 nM**:  
564 0.00054; **10 nM**:  $1.9 \cdot 10^{-11}$ ; **30 nM**:  $< 2 \cdot 10^{-16}$ ; **100 nM**:  $< 2 \cdot 10^{-16}$ . (N=14; 14; 14; 14; 14)

565 P-values baseline versus dose. Panel b i) **1 nM**: 1; **3 nM**: 1; **10 nM**: 1; **30 nM**: 1; **100 nM**: 1; **300 nM**:  
566 1. Panel b ii) **1 nM**: 1; **3 nM**: 1; **10 nM**: 1; **30 nM**: 1; **100 nM**: 1; **300 nM**: 1. (N=6; 5; 6; 6; 6; 6; 6)

567 Panel b iii) **3 nM**: 1; **10 nM**: 1; **30 nM**: 1; **100 nM**: 0.00801; **300 nM**:  $2.7 \cdot 10^{-9}$ ; **1000 nM**:  $1.8 \cdot 10^{-10}$ .

568 Panel b iv) **3 nM**: 1; **10 nM**: 1; **30 nM**: 1; **100 nM**: 0.00084; **300 nM**:  $2.9 \cdot 10^{-11}$ ; **1000 nM**:  $1.5 \cdot 10^{-11}$ .  
569 (N=6; 6; 6; 6; 6; 6; 6)

570 P-values baseline versus dose. Panel c i) **1 nM**: 1; **3 nM**: 1; **10 nM**: 1; **30 nM**: 1; **100 nM**: 1; **300 nM**:  
571 1. Panel c ii) **1 nM**: 1; **3 nM**: 1; **10 nM**: 1; **30 nM**: 1; **100 nM**: 1; **300 nM**: 1. (N=5; 5; 4; 5; 4; 4; 4)

572 Panel c iii) **3 nM**: 1; **10 nM**: 1; **30 nM**: 1; **100 nM**: 0.00181; **300 nM**:  $2.9 \cdot 10^{-6}$ ; **1000 nM**:  $1.7 \cdot 10^{-5}$ .  
573 Panel c iv) **3 nM**: 1; **10 nM**: 1; **30 nM**: 1; **100 nM**: 0.54836; **300 nM**: 0.01392; **1000 nM**:  $8.2 \cdot 10^{-5}$ .  
574 (N=5; 5; 4; 5; 5; 5; 3)  
575 P-values baseline versus dose. Panel d i) **1 nM**: 1; **3 nM**: 1; **10 nM**: 1; **30 nM**: 0.47; **100 nM**: 1. Panel  
576 d ii) **1 nM**: 0.02318; **3 nM**: 0.00170; **10 nM**: 0.00028; **30 nM**: 0.00044; **100 nM**: 0.00113. (N=5; 5; 5;  
577 5; 5; 5). Panel d iii) **3 nM**: 1; **10 nM**: 1; **30 nM**: 1; **100 nM**:  $3 \cdot 10^{-5}$ . Panel d iv) **3 nM**: 1; **10 nM**:  
578 0.49856; **30 nM**: 0.01473; **100 nM**:  $7 \cdot 10^{-6}$ . (N=6; 6; 6; 6; 6)  
579 P-values Krebs versus dose. Panel e i) **1 nM**: 1; **3 nM**: 1. Panel e ii) **1 nM**: 1; **3 nM**: 0.54. (N=6; 10; 7)  
580 P-values DMSO versus dose. Panel e iii) **10 nM**: 1; **30 nM**: 1; **100 nM**: 1; **300 nM**: 1. Panel e iv) **10**  
581 **nM**: 0.5298; **30 nM**: 0.2470; **100 nM**: 0.0054; **300 nM**: 0.0029. (N=7; 8; 4; 5; 7).

582

## 583 **Figure 5**

### 584 **In vivo disease phenotypes.**

585 **a)** Representative examples of wild type (**top**) and *gnb5a/gnb5b* mutant (**bottom**) zebrafishes and  
586 relative enhancement of moving pixels.

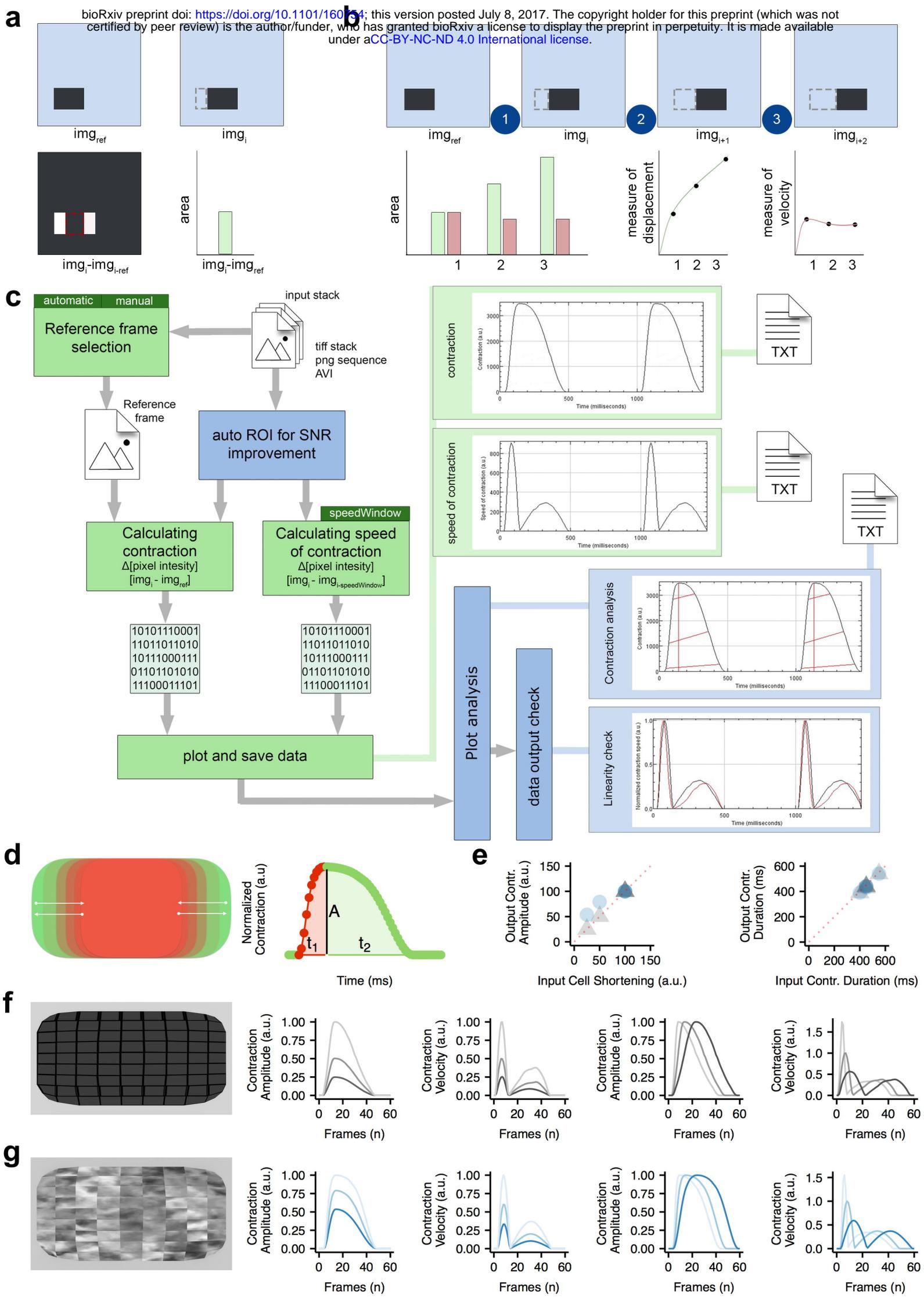
587 **b)** Representative qualitative analyses of normal (**top**) and arrhythmic (**bottom**) contraction profiles  
588 from wild type and *gnb5a/gnb5b* mutant zebrafishes treated with carbachol.

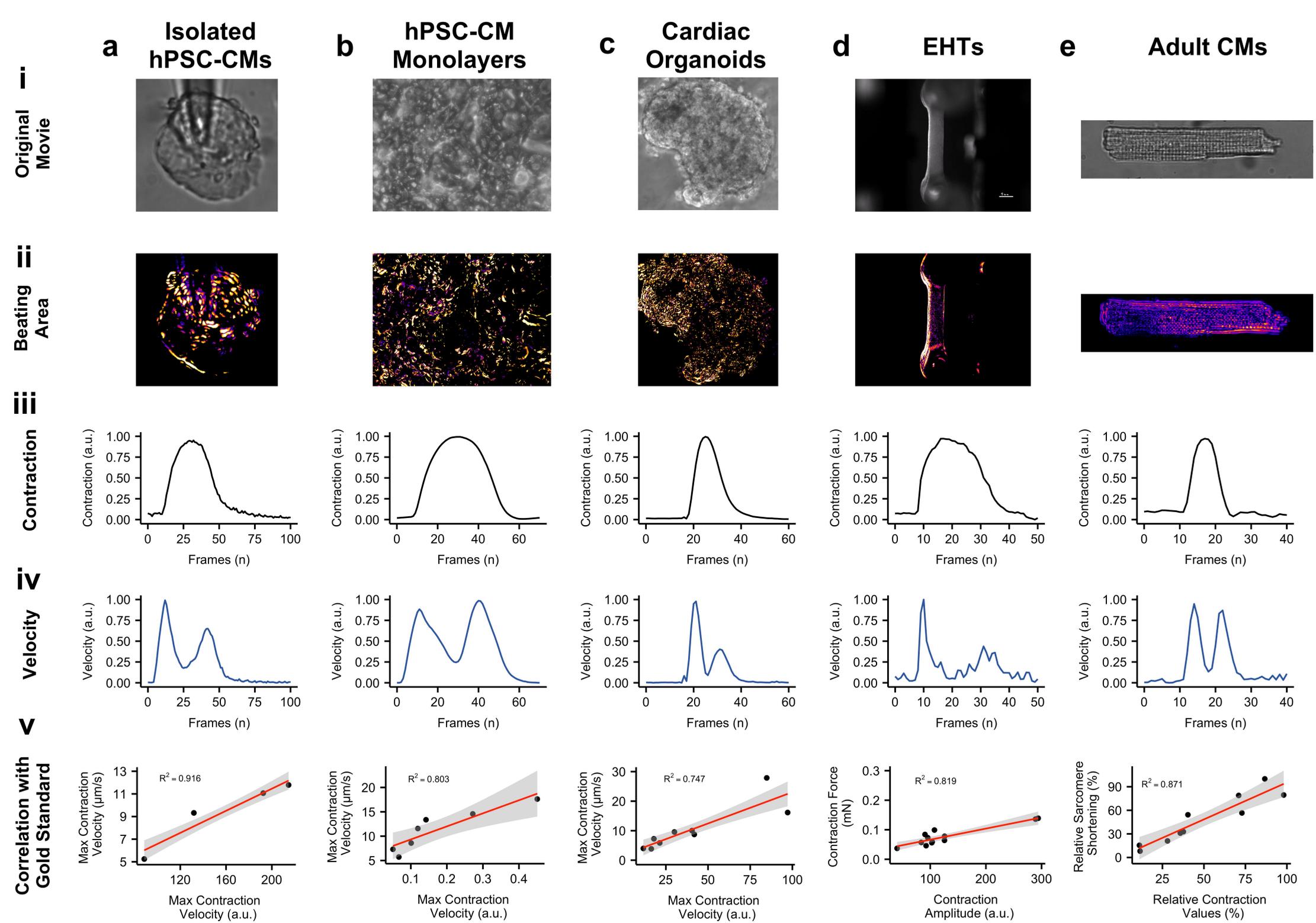
589 **c)** Correlation of results obtained from manual (x-axis) vs automatic (y-axis) detection of beating  
590 frequency (**top**); distribution of normal (green) and arrhythmic (red) contraction patterns in baseline  
591 condition (B) and after treatment with carbachol (C) in wild type and *gnb5a/gnb5b* mutant zebrafishes  
592 (**bottom**).

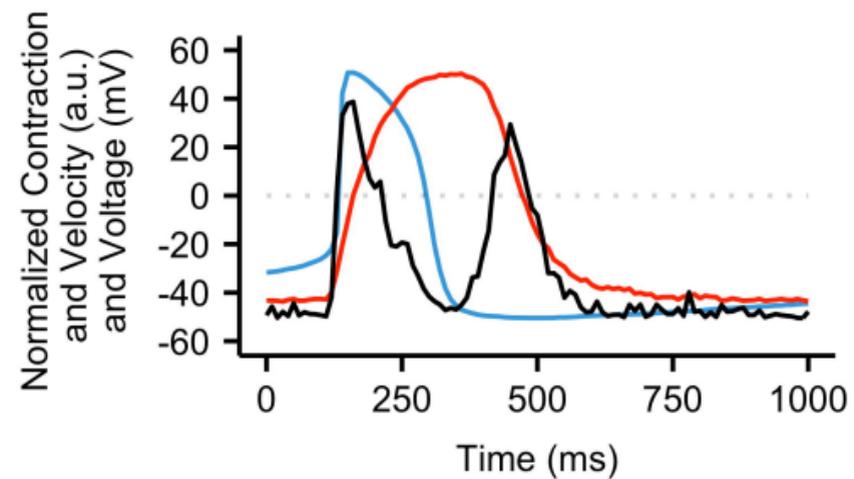
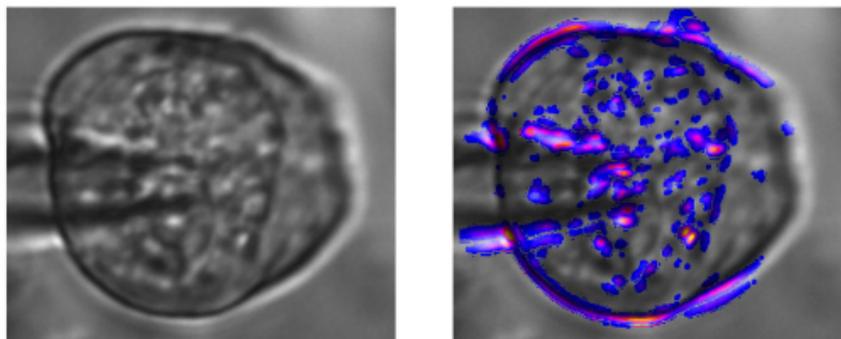
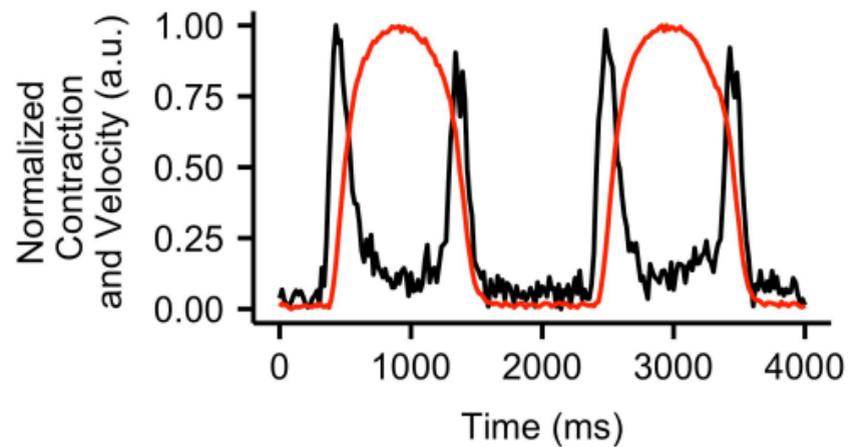
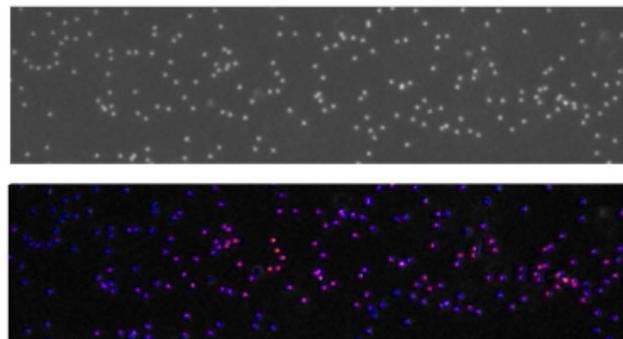
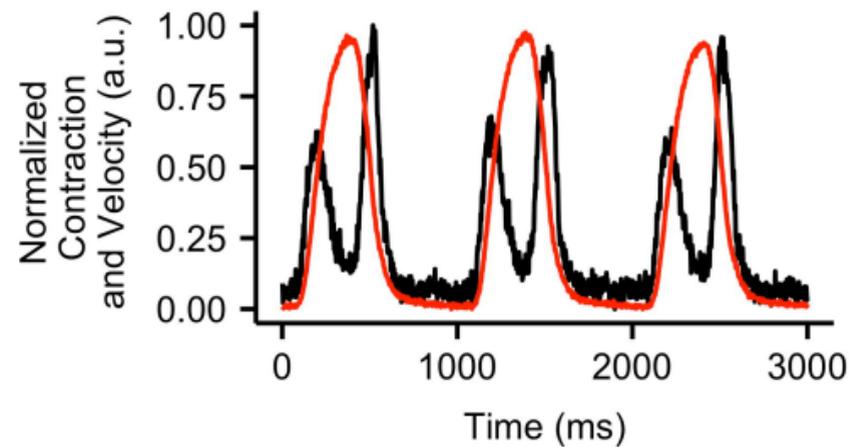
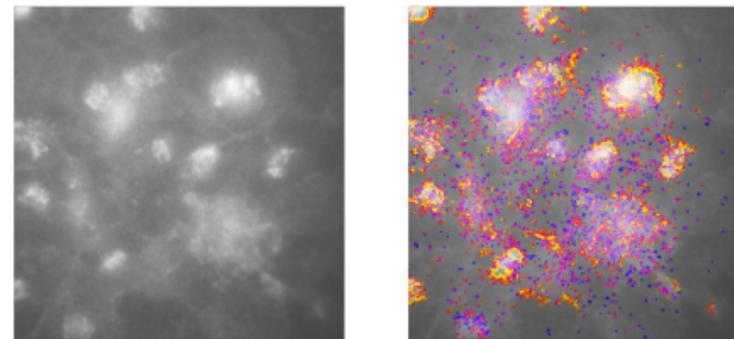
593 **d)** Representative echocardiograms of healthy (**top**) and cardiomyopathic (**bottom**) human  
594 individuals. Ventricles have been manually cropped and the enhancement of moving pixels is overlaid.

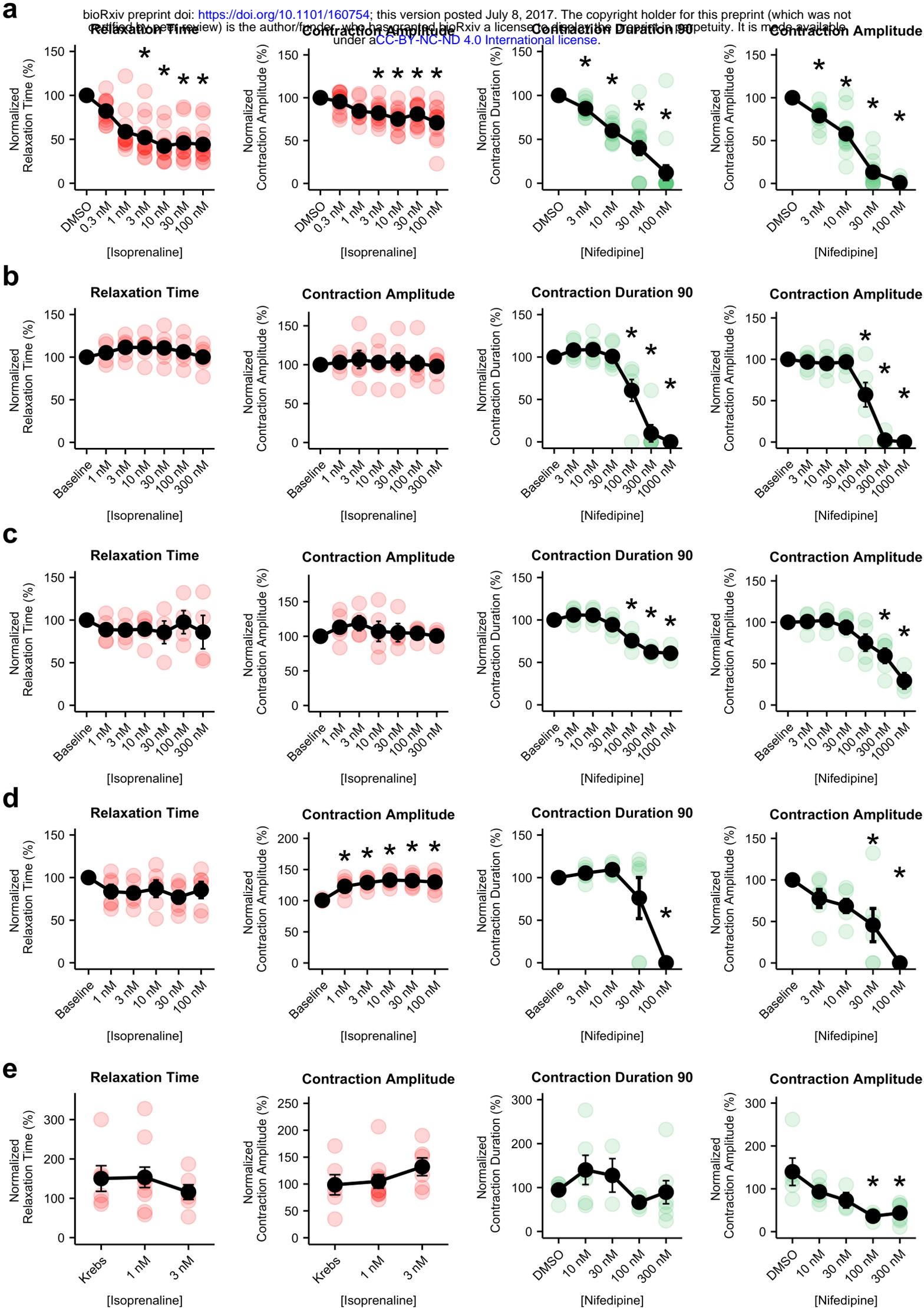
595 **e)** Representative qualitative analyses of normal (**top**) and poor (**bottom**) ventricular functions.

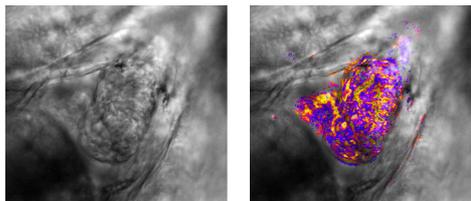
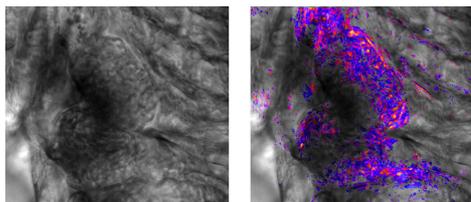
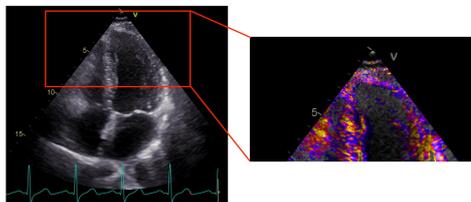
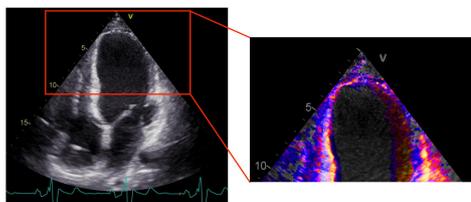
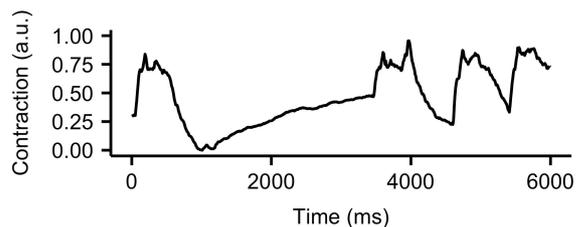
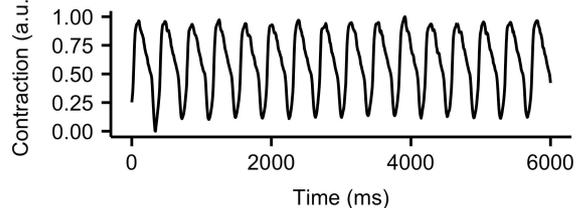
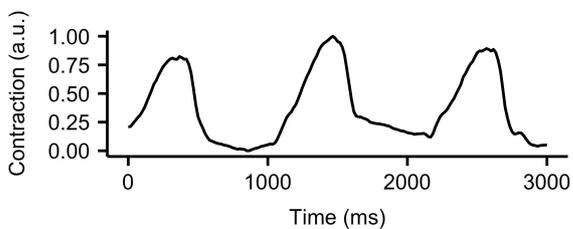
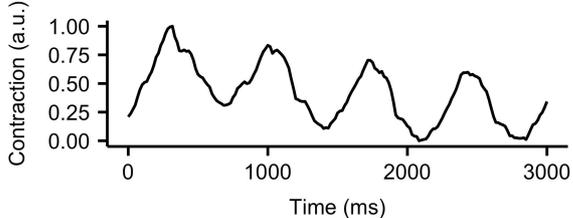
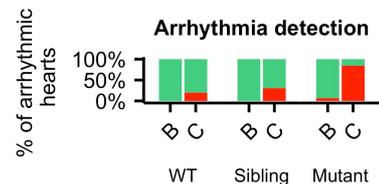
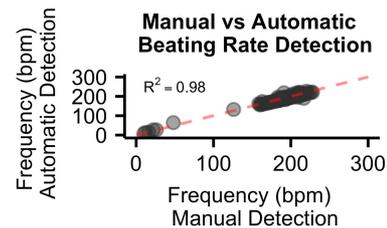
596 **f)** Quantitative data collected from echocardiogram in 5 individuals. Each colour represents one  
597 individual.





**a****Patch Clamp****b****Fluorescent Bead Tracking****c****Membrane Labelling**



**a****Healthy****Diseased****Ω****Healthy****Diseased****b****e****c****f**