

Intact myocardial preparations reveal intrinsic transmural heterogeneity in cardiac mechanics

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Pitoulis, F. G., Hasan, W., Papadaki, M., Clavere, N. G., Perbellini, F., Harding, S. E., Kirk, J. A., Boateng, S., de Tombe, P. P. and Terracciano, C. M. (2020) Intact myocardial preparations reveal intrinsic transmural heterogeneity in cardiac mechanics. *Journal of Molecular and Cellular Cardiology*, 141. pp. 11-16. ISSN 0022-2828 doi: <https://doi.org/10.1016/j.yjmcc.2020.03.007> Available at <https://centaur.reading.ac.uk/89907/>

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Published version at: <http://dx.doi.org/10.1016/j.yjmcc.2020.03.007>

To link to this article DOI: <http://dx.doi.org/10.1016/j.yjmcc.2020.03.007>

Publisher: Elsevier

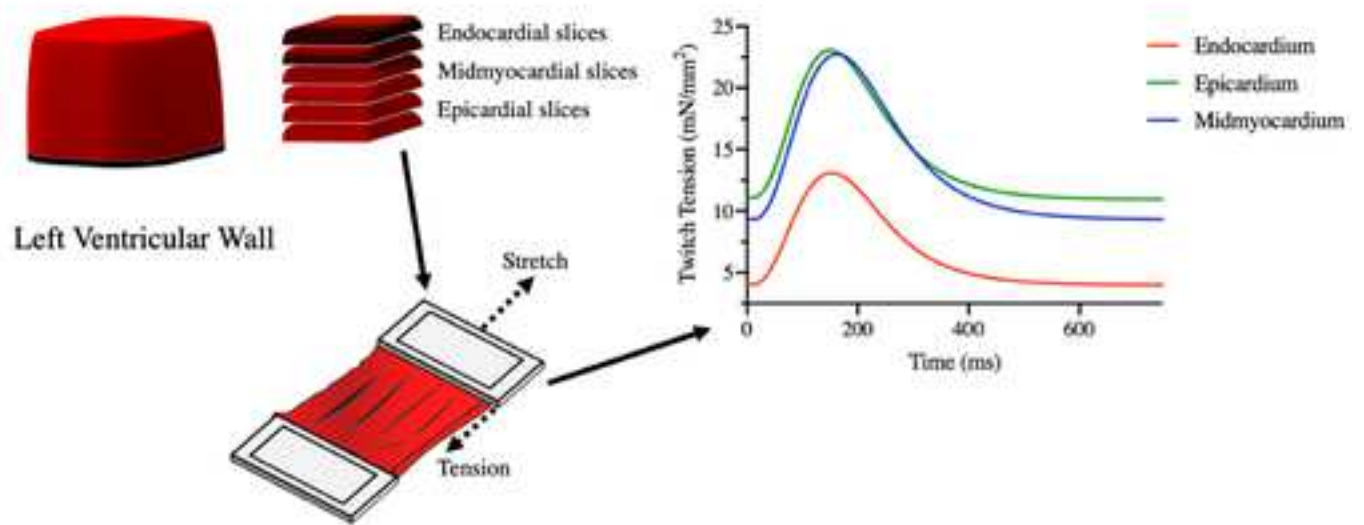
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1 Highlights

- 2 • The myocardial slice preparation is an intact cardiac model allowing the study of
- 3 transmural properties.
- 4 • Mechanical behaviour is cardiac layer dependent.
- 5 • Structural differences in cardiomyocyte density, orientation, and aspect ratio may
- 6 contribute to these findings.

1 Intact myocardial preparations reveal intrinsic transmural heterogeneity in cardiac mechanics

4 Authors List

5 Fotios G Pitoulis¹, Waseem Hasan¹, Maria Papadaki², Nicolas G Clavere³, Filippo Perbellini^{1, 4}, Sian E Harding¹, Jonathan A Kirk², Samuel Y Boateng³, Pieter P de Tombe^{1, 2}, Cesare M Terracciano¹

8
9 1: National Heart and Lung Institute, Imperial College London, UK

10 2: Department of Cell and Molecular Physiology, Loyola University Chicago, USA

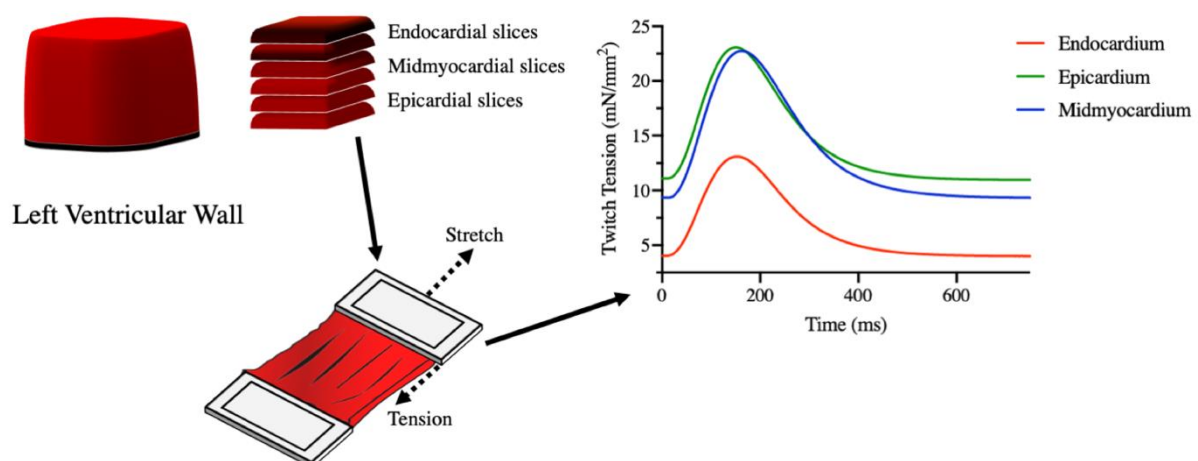
11 3: School of Biological Sciences, University of Reading, UK

12 4: Institute for Molecular and Translational Therapeutic Strategies, Hannover Medical School, DE

14 Abstract

15 Determining transmural mechanical properties in the heart provides a foundation to
16 understand physiological and pathophysiological cardiac mechanics. Although work
17 on mechanical characterisation has begun in isolated cells and permeabilised sam-
18 ples, the mechanical profile of living individual cardiac layers has not been exam-
19 ined. Myocardial slices are 300 μm -thin sections of heart tissue with preserved cellu-
20 lar stoichiometry, extracellular matrix, and structural architecture. This allows for
21 cardiac mechanics assays in the context of an intact *in vitro* organotypic preparation.
22 In slices obtained from the subendocardium, midmyocardium and subepicardium of
23 rats, a distinct pattern in transmural contractility is found that is different from that
24 observed in other models. Slices from the epicardium and midmyocardium had a
25 higher active tension and passive tension than the endocardium upon stretch. Differ-
26 ences in total myocyte area coverage, and aspect ratio between layers underlined the
27 functional readouts, while no differences were found in total sarcomeric protein and
28 phosphoprotein between layers. Such intrinsic heterogeneity may orchestrate the
29 normal pumping of the heart in the presence of transmural strain and sarcomere
30 length gradients in the *in vivo* heart.

32 Graphical Abstract



34

35 Highlights

- 36 • The myocardial slice preparation is an intact cardiac model allowing the study of
- 37 transmurality properties.
- 38 • Mechanical behaviour is cardiac layer dependent.
- 39 • Structural differences in cardiomyocyte density, orientation, and aspect ratio may
- 40 contribute to these findings.

41

42 Keywords

- 43 • Transmurality
- 44 • Contractility
- 45 • Cardiac Mechanics
- 46 • Intact Tissue
- 47 • Sarcomeric Apparatus
- 48 • Extracellular Matrix

49 1. Introduction

50 The ventricular myocardium is increasingly recognized as a structure with re-
51 gional variation. Although differences in electrical properties across the heart wall
52 have been the subject of multiple studies, descriptions of transmural mechanical
53 properties are marred by a paucity of data[1][2].

54 Determining mechanical behaviour across the heart wall is important as transmu-
55 ral differences in contractility can impact cardiac stroke volume, and changes in
56 transmural differences have been observed in human heart failure[2].

57 However, data on transmural mechanical behavior has been conflicting. Ambigu-
58 ity remains with respect to whether the endocardium has a higher passive tension
59 (i.e. is stiffer)[3][4] and active tension[3] than the epicardium or not[2][5][6]. Addi-
60 tionally, studies have been limited to isolated cardiomyocytes and permeabilised
61 preparations. Such cardiac models provide insight into the cellular and subcellular
62 basis of transmural differences but are restricted in their ability to capture the properties of
63 the *in-situ* myocardium. Isolated cardiomyocytes assess single cells independent of
64 the effect of intercalated discs, extracellular matrix (ECM), multi- & hetero-cellular-
65 ity in regulating contraction[7], and the transmural variation of these. Likewise, in
66 permeabilised samples, sarcolemmal components and the ECM - both known to af-
67 fect contractility - are disrupted. Therefore, whether transmural differences observed
68 in these models translate to intact living tissue remains unanswered.

69 Myocardial slices are a cardiac model of intermediate complexity serving as a
70 bridge between isolated cells and whole heart studies. Slices are 300 μm -thin living
71 organotypic preparations with native cellular architecture, cell-cell/cell-ECM inter-
72 actions, and preserved metabolic, electric, and mechanical properties[7]. Thus, slices
73 are a novel intact physiological model on which to evaluate cardiac behavior. The
74 slice model permits force vectors to be examined across a 2D plane, enabling me-
75 chanical insights comparable to those conducted on isolated cells and permeabilised
76 preparations. Transmural cardiac mechanics were assessed in rat slices and the un-
77 derlying structural, and sarcomeric differences explored.

78 2. Methods

79 2.1. Myocardial Slice Preparation

80 Myocardial slices were generated as described in [7]. Briefly, slices were pre-
81 pared from the left ventricles of 11-15-week-old Sprague-Dawley rats (350-470 g)
82 using a high-precision vibratome. Slices were generated in sequence, from endocar-
83 dium to epicardium. Most ventricles yielded six slices. The earliest slice of 300 μm
84 thickness, after removal of trabeculae carneaе, was defined as the first slice. The 1st
85 and 2nd slices were classified as subendocardial myocardium, the 3rd and 4th slice as
86 midmyocardium, and the 5th and 6th slices as subepicardial myocardium. For clarity,
87 these will be referred to as endocardium, midmyocardium and epicardium.

88

89 2.2. Laser Diffraction Experiments

90 The % stretch-sarcomere length relationship was determined using laser diffrac-
91 tion. A high-powered laser was vertically directed on slices, which were progres-
92 sively stretched, and the diffraction profiles processed in real-time (ImageJ, NIH,
93 USA).

94

95 2.3. Contractility Measurements

96 Contractility was assessed by mounting the slice on an isometric strain gauge
97 (F30 Harvard Apparatus, UK), using custom 3D-printed holders glued on the slice.
98 As myofiber direction rotates across the ventricular wall, holders were always at-
99 tached perpendicular to main myofiber axis. All slices were stimulated at 1Hz.

100

101 2.4. Sarcomeric Protein Content and Phosphorylation Status

102 Myofilament fractions were generated as previously described[8] from endo-,
103 mid-, and epicardial slices. Total sarcomeric content and phosphoprotein status were
104 quantified using Sypro Ruby and ProQ Diamond stain respectively.

105

106 2.5. Immunohistochemistry and Picrosirius Red Staining

107 Cardiac slices were fixed and stained for caveolin-3, cardiac troponin T, and vi-
108 mentin, and visualized under confocal microscopy within 1 day of staining. For col-
109 lagen content hearts were cryosectioned, fixed, stained with Picrosirius red, and vis-
110 ualized under brightfield microscopy.

111

112 2.6. Data Analysis

113 Contractility measurements were analysed using pClamp software (Molecular De-
114 vices, USA). Confocal and bright field images, as well as gels were analysed in Im-
115 ageJ.

116

117 2.7. Statistical Analysis

118 Data sets from each layer were analysed for statistical significance using AN-
119 COVA and one-way ANOVA with Tukey's post-hoc test in Prism 8 (GraphPad,
120 USA). $P < 0.05$ was considered statistically significant.

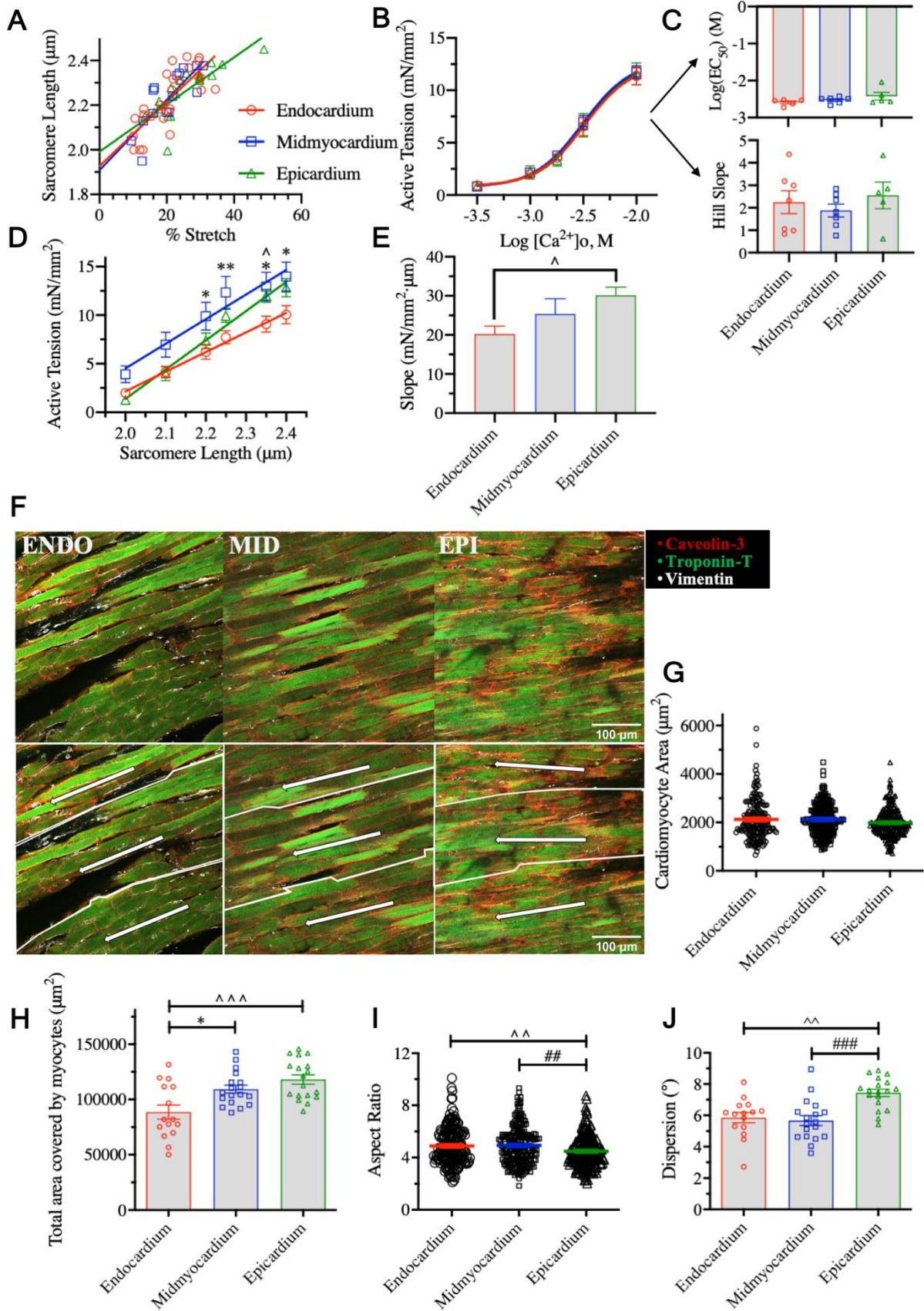
121 A detailed description of the methodology is available in the online supplement.

122 3. Results and Discussion

123 Cardiac layers have been suggested to operate at different sarcomere lengths (SL)
124 *in vivo*[3][9]. We identified the relationship between % stretch from slack length and
125 SL change (Fig. 1A) using laser diffraction. This allowed control of SL, so that in-
126 trinsic differences of each cardiac layers to the same strain could be delineated.

127 As force production is a function of external Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$), differ-
128 ent force sensitivity to $[\text{Ca}^{2+}]_o$ would mask intrinsic contractile differences. To ac-
129 count for this, we conducted a series of force- $[\text{Ca}^{2+}]_o$ experiments with slices
130 stretched to $2.1\mu\text{m}$ SL (Fig. 1B). Ca^{2+} sensitivity, defined as the $[\text{Ca}^{2+}]_o$ at which
131 half-maximum force is generated (EC_{50}), was not significantly different between lay-
132 ers (Fig. 1C-Top). It is worth noting that this EC_{50} is a different parameter to the one
133 reported in permeabilised preparations, which describes the response of intracellular
134 components to Ca^{2+} . Permeabilised human endocardial preparations for example, ex-
135 hibit greater Ca^{2+} sensitivity than the epicardium [2]. In contrast, our data suggests
136 that the Ca^{2+} sensitivity of the intact tissue is homogeneous across the wall. Moreo-
137 ver, the Hill slope, which measures the steepness of the force- $[\text{Ca}^{2+}]_o$ curve, was not
138 different between layers, further supporting a uniform contractile response to Ca^{2+}
139 (Fig. 1C-Bottom). These experiments yielded a known Ca^{2+} concentration ($\text{EC}_{50} =$
140 $10^{-2.54}$ M) at which the mechanical properties of the heart could be reliably assessed
141 while controlling for $[\text{Ca}^{2+}]_o$.

142 To determine contractile profiles across the wall, Frank-Starling experiments
143 were performed at a range of SLs ($2.00\text{--}2.40\mu\text{m}$). The midmyocardium produced a
144 significantly higher active tension than the endocardium at SLs $2.20\text{--}2.40\mu\text{m}$
145 ($p < 0.05$ and $p < 0.01$). Higher isometric force and power output has similarly been re-
146 ported in permeabilised midmyocardium [2]. The epicardium also trended towards
147 higher force development compared to the endocardium (Fig. 1D, $p < 0.05$ at SL of
148 $2.35\mu\text{m}$). Contrasting data exists regarding active tension between these latter two
149 layers and results appear to be species-dependent; in ferrets, the endocardium is re-
150 ported to have a higher maximum active tension than the epicardium[3] whereas in
151 pigs[6] and, more comparably, in rats no differences are described[3]. To quantify
152 the ability of each layer to increase its force output upon stretch, a linear regression
153 was fit to the active tension-SL relationship (Fig. 1D). The slope of this was signifi-
154 cantly higher in the epicardium compared to endocardium (Fig. 1E, $p < 0.05$), sug-
155 gesting greater contractile output per μm of stretch. When visualized under light mi-
156 croscopy, slices from the endocardium appear patchier and with recurring gaps be-
157 tween myofibers compared to other layers. Thus, we hypothesized that differences in
158 the cellular composition and tissue architecture may exist and play a role in shaping
159 the observed transmural mechanical heterogeneity.



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Figure 1: A) Transmural strain-sarcomere length (SL) relationship [N = 6]. B) Concentration-force response curves of cardiac slices from different layers to the external Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) [N = 8, N = 7, N = 6]. All force- Ca^{2+} experiments

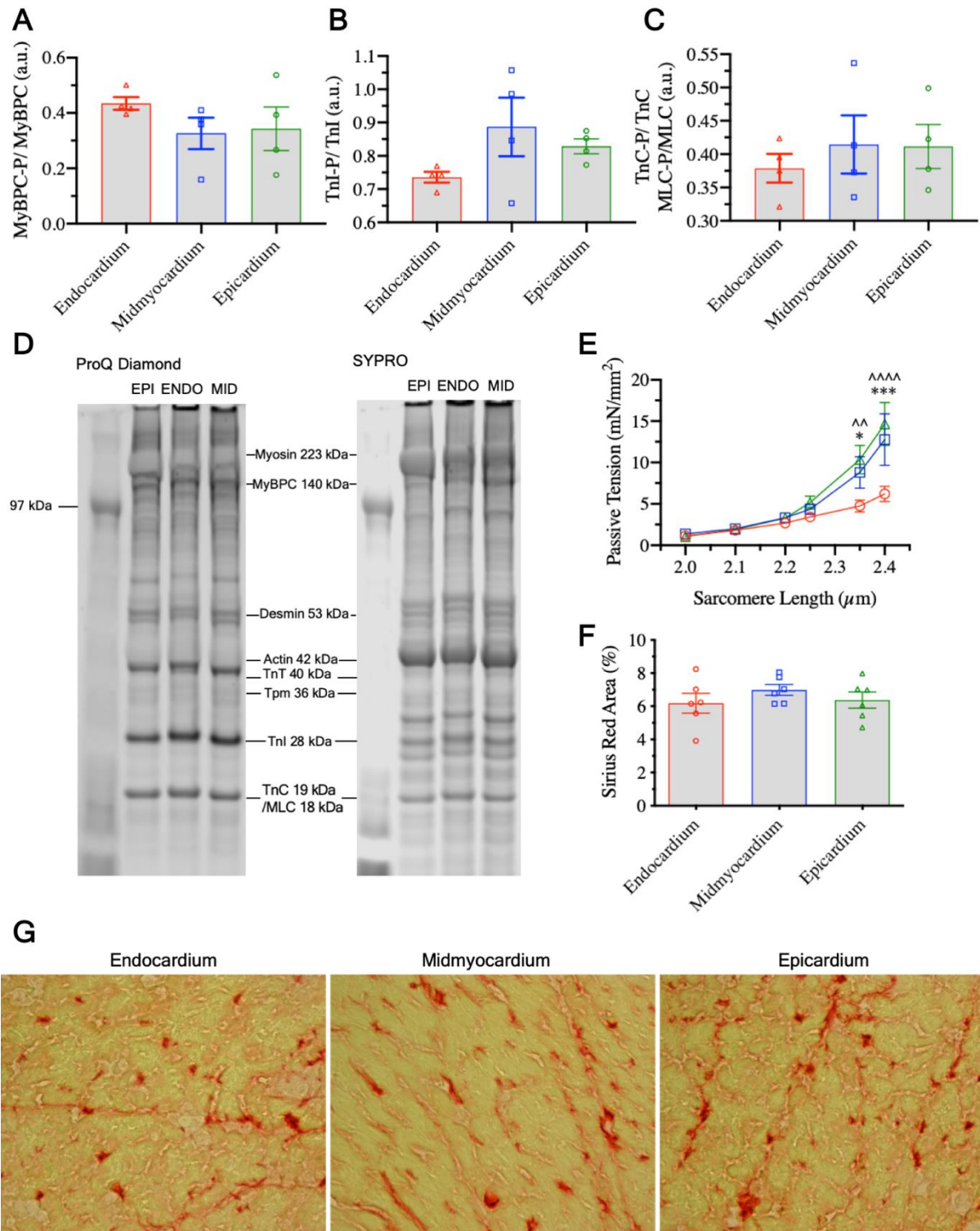
164 were conducted at 2.1 μm SL. C-Top) Transmural calcium sensitivity as measured
165 by the $\log(\text{EC}_{50})$ [N = 5, N = 6, N = 5]. The variable slope concentration-response
166 model fitted did not yield an EC_{50} for three endocardial, one midmyocardial, and one
167 epicardial data sets. C-Bottom) Hill coefficients of the concentration-response
168 curves of each cardiac layer [N = 7, N = 7, N = 5]. The variable slope concentration-
169 response model fitted yielded an ambiguous slope for one endocardial, and one epi-
170 cardial data set. D) Active tension-SL relationship for cardiac slices from different
171 layers of the wall [N = 7, N = 5, N = 7]. E) Slopes of linear regression lines fit to the
172 active tension-SL relationship [N = 7, N = 5, N = 7]. F) Representative confocal im-
173 ages of cardiomyocyte area, density, morphology, and direction. Each image in the
174 bottom panel has been separated into three regions and the major direction of cardio-
175 myocyte orientation drawn with an arrow. The greater the angle between the arrows
176 suggests the greater the variability in cardiomyocyte orientation. G) Cardiomyocyte
177 area [N = 180/5, N = 216/6, N = 216/6]. H) Total myocyte area coverage (myocyte
178 area \times number of cardiomyocytes) [N = 15/5, N = 18/6, N = 18/6]. I) Cardiomyocyte
179 aspect ratio [N = 180/5, N = 216/6, N = 216/6]. J) Cardiomyocyte dispersion [N =
180 15/5, N = 18/6, N = 18/6]. All analysis was done blinded. *: midmyocardium vs. en-
181 docardium, ^: epicardium vs. endocardium, #: midmyocardium vs. epicardium. [N =
182 endocardium, midmyocardium, endocardium, and N = images/biological replicates].
183

184 Cardiomyocyte area, which has been reported to be higher in isolated rat endocar-
185 dial than epicardial myocytes[10], was quantified (Fig. 1F-G). Although no differ-
186 ences were found in individual myocyte area, total myocyte coverage of the tissue
187 (myocyte area \times number of myocytes) was significantly higher in both midmyocar-
188 dium and epicardium compared to endocardium ($p < 0.05$, and $p < 0.001$ respectively)
189 (Fig. 1H). Cardiomyocyte area is a major determinant of systolic force production
190 and cardiomyocyte density correlates positively with force production in engineered
191 heart tissue[11]. An absolute greater myocyte coverage would thus explain the in-
192 creased active tension-SL relationship and steeper force-stretch response of the mid-
193 myocardium and epicardium. However, cardiomyocyte morphology can also impact
194 cardiac contraction with long and thin myocytes at a mechanical disadvantage when
195 generating force compared to shorter and thicker cells[12]. The cardiomyocyte as-
196 pect ratio (length:width) was significantly lower in epicardial cells compared to both
197 midmyocardium and endocardium (Fig. 1I, $p < 0.01$).

198 Despite the lower aspect ratio, the force-stretch experiments showed that midmyo-
199 cardium produces a higher active tension compared to the endocardium more con-
200 sistently than the epicardium, and does so at a lower SL. We hypothesized that car-
201 diomyocyte orientation could account for this. Cardiomyocyte dispersion, a measure
202 of the standard deviation of a Gaussian curve fitted to the different angulations of
203 structures (i.e. cardiomyocytes) in an image from the main axis of direction was
204 quantified. Dispersion was significantly higher in the epicardium compared to endo-
205 cardium and midmyocardium, suggesting greater cardiomyocyte orientation variabil-
206 ity (Fig. 1J, $p < 0.01$ and $p < 0.001$ respectively). As myocyte misalignment can reduce

207 force development[13], higher cardiomyocyte disorientation may offset force pro-
208 duction in the epicardium despite the lower aspect ratio and explain why the mid-
209 myocardium tends to develop marginally higher active tension than the epicardium
210 when compared to the endocardium (Fig. 1D-E).

211 The sarcomeric apparatus could also underlie transmural differences in active ten-
212 sion. As such, we performed total sarcomeric protein content and phosphoprotein
213 status quantification using Sypro Ruby and ProQ Diamond staining. However, our
214 results showed no significant differences across the wall despite a gradient tendency
215 of certain proteins (Fig. 2A-D). Comparable transmural uniformity of sarcomeric
216 proteins has been reported in the non-failing human heart[2] and rats of similar age
217 by others[14].



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Figure 2: A-C) Ratio of phosphorylated myosin binding protein C, troponin I, and troponin C & myosin light chain to unphosphorylated counterpart respectively [N = 4]. D) Bands from total sarcomeric content (right) and phosphoproteins (left) separated by molecular weight. E) Passive tension-SL relationship for cardiac slices from different layers of the wall [N = 7, N = 5, N = 7]. F) % Area covered by Sirius red staining from transverse cryosections [N = 6]. G) Representative brightfield images of Sirius red staining. *: midmyocardium vs. endocardium, ^: epicardium vs. endocardium. [N = endocardium, midmyocardium, endocardium].

227

228 Passive tension was significantly lower in the endocardium compared to midmyo-
229 cardium and epicardium at 2.35 μm and 2.40 μm SL (Fig. 2E, $p < 0.001$ and $p < 0.0001$
230 at SL of 2.40 μm). In intact isolated cardiomyocytes, passive tension increases more
231 in the endocardium than epicardium with SL[3]. Data from skinned samples simi-
232 larly show a stiffer endocardium at high SLs[4], although others have reported no
233 differences between the two[2][5] nor between the midmyocardium and the other
234 layers[2]. Our slice data show that intact endocardial layers are less stiff.

235 Passive tension is particularly important during the refilling phase of the cardiac
236 cycle. Reconstruction of *in vivo* transmural SL gradients using radiopaque beads and
237 biplane cineradiography demonstrate that the endocardium operates at a higher and
238 narrower SL range than the mid- and epicardium, that can reach 2.4 μm length,
239 where our significant differences arise, prior to ejection[9]. Likewise, the loading
240 cycle of the isolated arrested heart shows clear transmural deformations (i.e. normal
241 strains – longitudinal, radial, and circumferential – increase from epicardium to en-
242 docardium)[15]. The consequences of a) transmural strain gradients, but b) uniform
243 SL-stretch relationships (Fig. 1A) in the presence of a more compliant endocardium
244 (Fig. 2E) is a greater deformation of the inner myocardium enabling it to attain a
245 higher SL. Under this conceptual framework, lower compliance of outer layers
246 would similarly facilitate their operation at lower SLs while averting excessive en-
247 docardial diastolic strain, in effect acting as ‘guardian’ layers.

248 These conclusions are important not only for diastole but also active cardiac con-
249 traction. Transmurally non-uniform SLs would position the endocardium further up
250 and to the right of the Frank-Starling curve and the outer layers further down and to
251 the left. Intrinsic differences in contractility (Fig. 1A-B), largely attributed here to
252 structural heterogeneity and total myocyte coverage (Fig. 1G-J), may thus be offset,
253 homogenizing force production and ventricular pumping efficiency[1]. In support of
254 this, cardiomyocytes with low aspect ratio develop the highest systolic work in stiff
255 environments whereas those with higher aspect ratio fare better in less stiff environ-
256 ments[16], which is in agreement with our functional and structural data.

257 To uncover the structural factors involved in differential passive tension we per-
258 formed transverse sectioning of tissue followed by picrosirius red staining. We
259 found no significant differences in total collagen content between layers (Fig. 2F-G).
260 Although collagen is a known determinant of passive myocardial mechanics, stiff-
261 ness is dominated by titin content and isoform composition. In the adult rat, titin, a
262 giant molecular sarcomeric protein, exists either as larger compliant N2BA or
263 smaller stiffer N2B isoform. Although we were unable to quantify this, others have
264 reported extensively that in healthy canine and pig hearts, the N2BA:N2B ratio
265 shows a transmural gradient highest in the subendocardium[17], consistent with our
266 mechanical data. Transmural heterogeneity in cross-bridge cycling could also under-
267 lie the observed differences. Testing this aspect would be insightful and will be part
268 of future investigations.

269 A number of limitations are discussed. First, myocardial slices generate stress and
270 respond to strain across a 2D-plane. This is in contrast to the *in vivo* 3D operation of

271 the heart, where pressure is generated corresponded with distinct changes in volume.
272 Additionally, fiber alignment is known to rotate across the 3D ventricular wall, in
273 contrast to the uniaxial plain of examination in slices. As slices from adjacent layers
274 (1st and 2nd slice & 5th and 6th slice) were collapsed together for data analysis, trans-
275 mural differences may have been underestimated. Another limitation is the use of
276 muscle length control for isometric measurements, which may introduce experi-
277 mental error due to damaged end-compliance[18]. However, the tension-SL relation-
278 ship with muscle length has been suggested to be the same as that of SL control[19].
279 Our data supports that functional differences in transmural mechanics are dominated
280 by structural heterogeneity and not sarcomeric protein content or phosphorylation
281 status. Transmural gradients in myosin light chain phosphorylation have been re-
282 ported [20] and suggested to explain the pattern of active mechanical contraction,
283 which is dominated by the outer layers. However, our results like those of others did
284 not show the presence of such spatial gradients[21].

285 In conclusion, we show for the first time that in intact tissue, intrinsic differences
286 exist in myocardial active and passive mechanics that are primarily governed by
287 structural transmural heterogeneity. *In vivo*, operation of cardiac layers at different
288 SLs has been shown and our findings provide a physiological explanation for this;
289 differences in operating SL seem to be balanced out by differences in the intrinsic
290 mechanical properties.

291 4. Supplementary Material

292 4.1. Myocardial Slice Preparation

293 All animal experiments were conducted in accordance with institutional and na-
294 tional regulations and were approved by Imperial College London, under license by
295 the UK Home Office, United Kingdom Animals (Scientific Procedures) Act 1986.

296 Myocardial slices were prepared using the protocol previously detailed by Watson
297 et al.[7]. Adult Sprague-Dawley rats (aged 11-15 weeks and 370-450g weight) were
298 sacrificed under anaesthesia (4% isoflurane at 4 L/min oxygen), their heart ex-
299 planted and placed in 50 ml of heparinized (1000IU/ml) modified Tyrode solution
300 (30.0 mM 2, 3-Butanedione Monoxime, 140.0 mM NaCl, 9.0 mM KCl, 10 mM Glu-
301 cose, 10.0 mM HEPES, 1.0 mM Magnesium Chloride, 1.0 mM Calcium Chloride; pH
302 7.40) at 37°C and shortly thereafter at 4°C heparinized modified Tyrode solution.
303 The left ventricle was isolated by removing the lungs and other extra-cardiac struc-
304 tures using a surgical blade and dissecting away the atria and right ventricle. It was
305 then opened up, flattened and the epicardial side glued (Histoacryl®, Braun, Ger-
306 many) onto a layer of agarose (4% agar) on a specimen holder. The specimen holder
307 was mounted to the tissue bath of a high precision vibrating microtome (7000 smz2,
308 Campden Instruments Ltd., UK) previously filled with oxygenated 4°C modified
309 Tyrode solution.

310 Before slicing, the vibratome was fitted with a ceramic blade, calibrated to a Z-
311 axis error <1.0 µm and the blade adjusted to have an amplitude of 2 mm, vibration
312 frequency of 80 Hz and advance speed of 0.03 mm/s. These settings were chosen to
313 produce highly viable 300 µm myocardial slices. Each slice generated was examined
314 under light microscopy to determine myocardial fiber orientation. One section with
315 parallel fiber orientation and from the mid-region of each slice was selected and cut
316 into a rectangular sample. The latter was important to minimize apico-basal differ-
317 ences which could confound the results. The length and width of the slice were
318 measured using calipers and recorded for normalization of force to the slice cross-
319 sectional area. For slices used in the contractility, laser diffraction experiments as
320 well as for fixation, custom-designed 3D-printed Polylactic acid (PLA) holders were
321 attached to both ends of the sample perpendicular to the fiber orientation using sur-
322 gical glue. These were used to manipulate and mount the slice to the force-trans-
323 ducer, laser diffraction, or tissue fixation set-ups.

324

325 4.2. Laser Diffraction Experiments

326 Myocardial slices were mounted on custom-made stainless-steel stretcher using the
327 3D printed holders. The stretcher was positioned into a glass dish, filled with modi-
328 fied Tyrode's solution at room temperature. A high powered HeNe laser (Lasos, Ger-
329 many) was positioned 2cm vertically above the slice. The laser was turned on and as
330 it passed through the slice, it diffracted into bands based on the amount of tissue
331 stretch. A camera (Logitech C920 HD) was used to analyze the diffraction profiles
332 in real-time using the plot-profile functionality of ImageJ (National Institute of
333 Health, USA). Each slice was progressively stretched (from slack length) to three

334 different muscle lengths, and the data were used to determine the % stretch-sarco-
335 mere length (SL) relationship. To account for SL inhomogeneity across the tissue,
336 the laser was directed at three different regions of the myocardial slice at each mus-
337 cle length, the SL determined, and then averaged for each stretch.

338 A linear regression model was fit to the % stretch-SL relationship for slices from
339 each cardiac layer. Given a known slice slack length and the linear relationship be-
340 tween stretch and SL, the muscle length correspondent with a desired SL could be
341 calculated. This was subsequently used for fine manipulation of strain in force-cal-
342 cium and Frank-Starling experiments.

343

344 4.3. Force-Calcium Experiments

345 Normal Tyrode's solutions (140.0 mM NaCl, 4.5 mM KCl, 10 mM Glucose, 10.0
346 mM HEPES, 1.0 mM Magnesium Chloride; pH 7.40) with calcium concentrations of
347 0.316 mM ($10^{-3.5}$ M), 1 mM (10^{-3} M), 1.8 mM ($10^{-2.7}$ M), 3.16 mM ($10^{-2.5}$ M), 10 mM
348 ($10^{-2.0}$ M) were prepared.

349 Myocardial slices were moved into an organ bath chamber and mounted on an iso-
350 metric strain gauge (F30 Harvard Apparatus, USA) using the PLA holders. The or-
351 gan bath contained oxygenated 1.8 mM Tyrode's solution at 25°C (starting tempera-
352 ture). Electrical stimulation was initiated, and the temperature of the solution slowly
353 raised until $36 \pm 0.5^\circ\text{C}$. Slices were stretched to a SL of 2.1 μm using muscle length
354 as a surrogate. A micromanipulator and video camera connected to and calibrated
355 with the ImageJ software (National Institute of Health, USA) webcam plugin were
356 used for real-time measurements of muscle-length.

357 Slices were stimulated at 1 Hz, 20-30V amplitude and 10-20 ms width biphasic
358 pulses and data was acquired using AxoScope software (Molecular Devices, USA).
359 After one minute of steady state force production was achieved, perfusion of the cur-
360 rent solution was replaced with a randomly chosen Tyrode's solution containing an
361 unused calcium concentration. This process was repeated until measurements from
362 all calcium concentrations were obtained. At the end, slices were again assessed at
363 1.8 mM Tyrode's solution and if the active tension generated was <60% of the active
364 tension at the first 1.8mM solution, slices were excluded from further analysis.

365 The pClamp software package (Molecular Devices, USA) was used to analyze
366 three force transients generated at the steady state of each calcium concentration
367 (force-calcium) and SL (Frank-Starling – see section 4.4.). The forces generated by
368 the slices were normalized to their cross-sectional area (slice thickness 0.3 mm x
369 slice width).

370 Active tension generated during the force-calcium experiments were plotted
371 against a log scale of Ca^{2+} concentration and a sigmoidal log(agonist) vs. response
372 (variable slope, four parameters) model was fitted according to the equation:

373

$$A = B + C \frac{1}{1 + 10^{((\log E_{c50} - \log \text{Ca}^{2+})) * H}}$$

374 where A is the active tension, B is the passive tension, C is the maximum force
375 generated, H is the hill coefficient and EC_{50} is the calcium concentration required to
376 reach half maximum active tension.

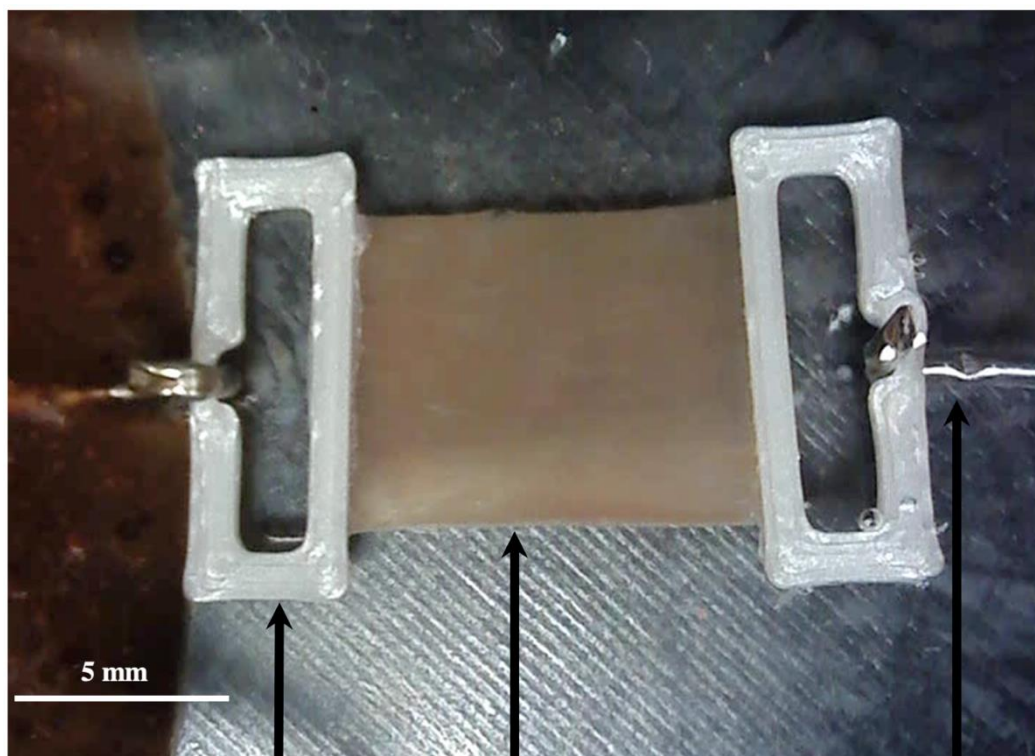
377

378 4.4. Frank-Starling Experiments (Active and Passive Tension at Different SL)

379 Myocardial slices were mounted onto the isometric strain gauge in the organ bath,
380 containing oxygenated 25°C Tyrode's solution but with $10^{-2.54}$ M Ca^{2+} (EC_{50}) and
381 slowly heated to $36 \pm 0.5^\circ C$. The slices were progressively stretched to muscle
382 lengths correspondent to SL of 2.0-, 2.10-, 2.20-, 2.25-, 2.35- and 2.40 μm . Slices
383 from different layers were stretched to each SL based on their individual SL-%
384 stretch profiles determined previously from the laser diffraction experiments. All
385 slices were field stimulated at 1 Hz, 20-30V amplitude and 10-20 ms width biphasic
386 pulses using platinum electrodes.

387 A linear regression was fit to the active tension-SL relationship; the gradient of
388 each line was compared to determine the force sensitivity of each cardiac layer to
389 stretch. R^2 values were 0.72, 0.60, and 0.84 for endocardium, midmyocardium, and
390 epicardium respectively.

391



392 PLA holders

393 Myocardial Slice

To Strain Gauge Micromanipulator

392

393

394 **Figure 1 Supplementary:** Myocardial slice inside the organ bath. 3D-printed PLA
 395 holders are attached perpendicular to the direction of myocardial fibers and used to
 396 mount the slice on an isometric strain gauge. The slice is stimulated with platinum
 397 electrodes (not visible here) at 1Hz and the active and passive tension recorded.

398

399 4.5. Immunohistochemical Staining for Confocal Imaging

400 Cardiac slices were mounted on stretchers, stretched to 2.1 μm SL, and fixed in 4%
 401 formaldehyde for 15 minutes at room temperature, washed in phosphate buffered so-
 402 lution (PBS, Sigma), permeabilised and blocked using 1.5% Triton X-100 in 10%
 403 FBS, 5% BSA and 10% horse serum for three hours at room temperature on a rocker.
 404 Slices were then washed in PBS three times and incubated with their primary anti-
 405 bodies overnight at room temperature on a rocker. After being washed a further three
 406 times in PBS, they were incubated with their secondary antibody for 2 hours at room
 407 temperature on a rocker, covered with aluminum foil. After a final three washes, the
 408 samples were stored at 4°C in PBS. All stained slices were visualized under confocal
 409 microscopy within 1 day of being stained. Myocardial slices were viewed under a
 410 confocal microscope (Zeiss LSM-780) and three Z-stack images were taken from
 411 each slice at 20x magnification.

412 **Table 1:** Antibodies used to stain cardiac slices

Antibody	How it is created	Dilution
Primary Antibodies		
Caveolin 3	Mouse	1:500
Cardiac Troponin T	Rabbit	1:800
Vimentin	Chicken	1:3000
Secondary Antibodies		
Alexa Fluor 488	Donkey, anti-rabbit	1:2000
Alexa Fluor 546	Donkey, anti-mouse	1:2000
Alexa Fluor 647	Goat, anti-chicken	1:2000

413

414 Confocal images were analyzed blinded in ImageJ. Caveolin-3 stained the mem-
 415 brane of the cardiomyocytes and allowed identification of the cells and their borders.
 416 Length of the cell was defined as the longest axis across a cardiomyocyte and width
 417 as the longest line perpendicular to the length axis. Cell area was measured by cir-
 418 cumscribing along the cardiomyocyte borders demarked by caveolin-3 staining. Cell
 419 density was quantified by counting the number of fully visible cells present in each
 420 image. 12 cells were analysed for length:width ratio, and area from each image. To-
 421 tal myocyte area was determined as the product of number of cardiomyocytes \times car-
 422 diomyocyte area. Vimentin levels were obtained from each image's first four Z-
 423 stacks and were normalized using the auto-threshold settings on ImageJ. They were
 424 subsequently measured using the pixel quantification tool. Dispersion was calculated
 425 using data from all Z-stacks available in the image and measured with the software's
 426 directionality analysis tool.

427

428 4.6. Sarcomeric Proteins

429 Myofilament fractions were prepared from endocardium, midmyocardium, and epi-
430 cardium as previously described[8]. Myofilament proteins (20 µg per lane) were run
431 on a 4-12 % gradient gel. The gel was then stained with ProQ Diamond according to
432 manufacturer's instructions (Invitrogen). Briefly, the gel was fixed in a 10% acetic
433 acid, 50% methanol solution for one hour. It was then stained with ProQ stain for
434 75 mins and destained with a solution containing 20% acetonitrile, 50 mM sodium
435 acetate, pH 4.00. The resulting gel was imaged using the Typhoon scanner (GE
436 Healthcare) at 580 nm. After imaging, it was stained overnight with Sypro Ruby
437 stain (Invitrogen) and after destain with 10% methanol, 7% acetic acid, it was im-
438 aged using the Typhoon scanner at 619 nm. Band quantification was performed us-
439 ing Image J and the ratio of each phosphorylated protein to total protein was calcu-
440 lated.

441

442 4.7. Picrosirius Red Staining

443 The harvested hearts of six SD rats were blocked in Optimal Cutting Temperature
444 media and stored at -80°C for 24 hours. 7 µm transverse sections were taken by cut-
445 ting the OCT blocks with a cryostat at -23°C and harvested on slides, before being
446 stored at -80°C. To visualize cardiac fibrosis, sections were stained with Picrosirius
447 Red staining kit (ab150681, Abcam). To do that, they were prefixed in Bouin solu-
448 tion for 15 minutes under fume hood at room temperature then washed for 15
449 minutes at room temperature in tap water. Then, sections were stained in Picrosirius
450 red for 1 hour at room temperature under the fume hood. Slides were differentiated
451 in acidified water, twice two dips. Sections were then washed and dehydrated three
452 times in 100% Ethanol before being cleared in Xylene over five minutes. Cover slips
453 were mounted, and imaging was done with brightfield microscope Nikon TE200. 10
454 pictures per area of interest per section were obtained, and two sections were ana-
455 lysed for each animal. The quantification of the red stain was carried out with Im-
456 ageJ. In a first time the original picture was changed to RGB format. Then, a thresh-
457 old previously calibrated in order to cover most of the staining was applied on the
458 RGB picture. After applying the threshold on the picture, the area covered by the
459 stain was measured.

460

461 4.8. Statistical Analysis

462 For comparison of statistical significance between linear regression lines (laser dif-
463 fraction and active tension-SL relationships) analysis of co-variance (ANCOVA)
464 was used. For contractility (active-tension, passive-tension, logEC₅₀, Hill slope) and
465 confocal images, the data sets of slices from each layer were compared using one
466 way analysis of variance (ANOVA) with Tukey's post-hoc in Prism8 software
467 (GraphPad, USA). P<0.05 was considered statistically significant.

468 5. Disclosures

469 Part of this study was featured as an abstract in the European Society of Cardiol-
470 ogy Congress, Paris 2019. All authors declare no conflicts of interest, financial or
471 otherwise.

472

473 6. Acknowledgements

474 We thank the British Heart Foundation for funding to Fotios Pitoulis under the
475 MBBS PhD studentship scheme (FS/18/37/33642).

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