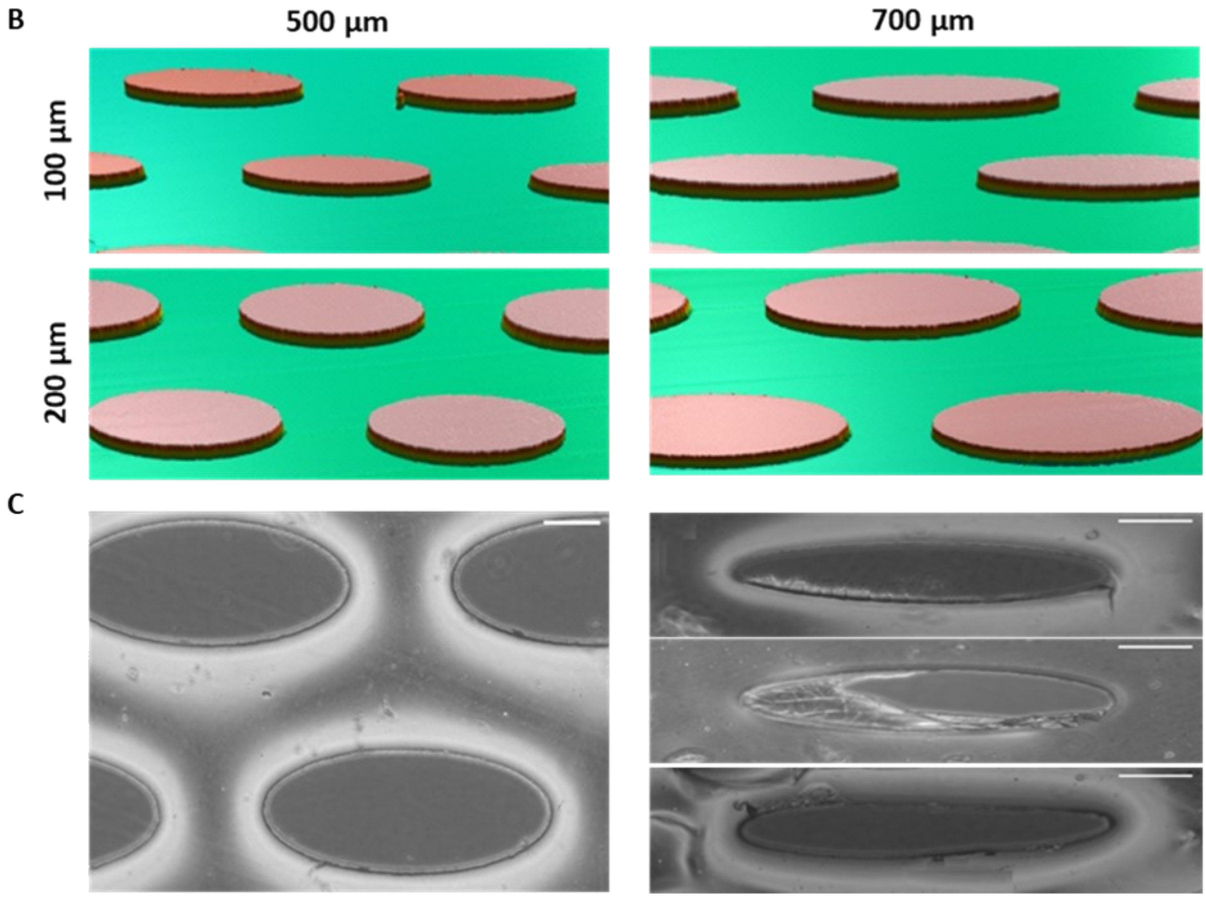
A

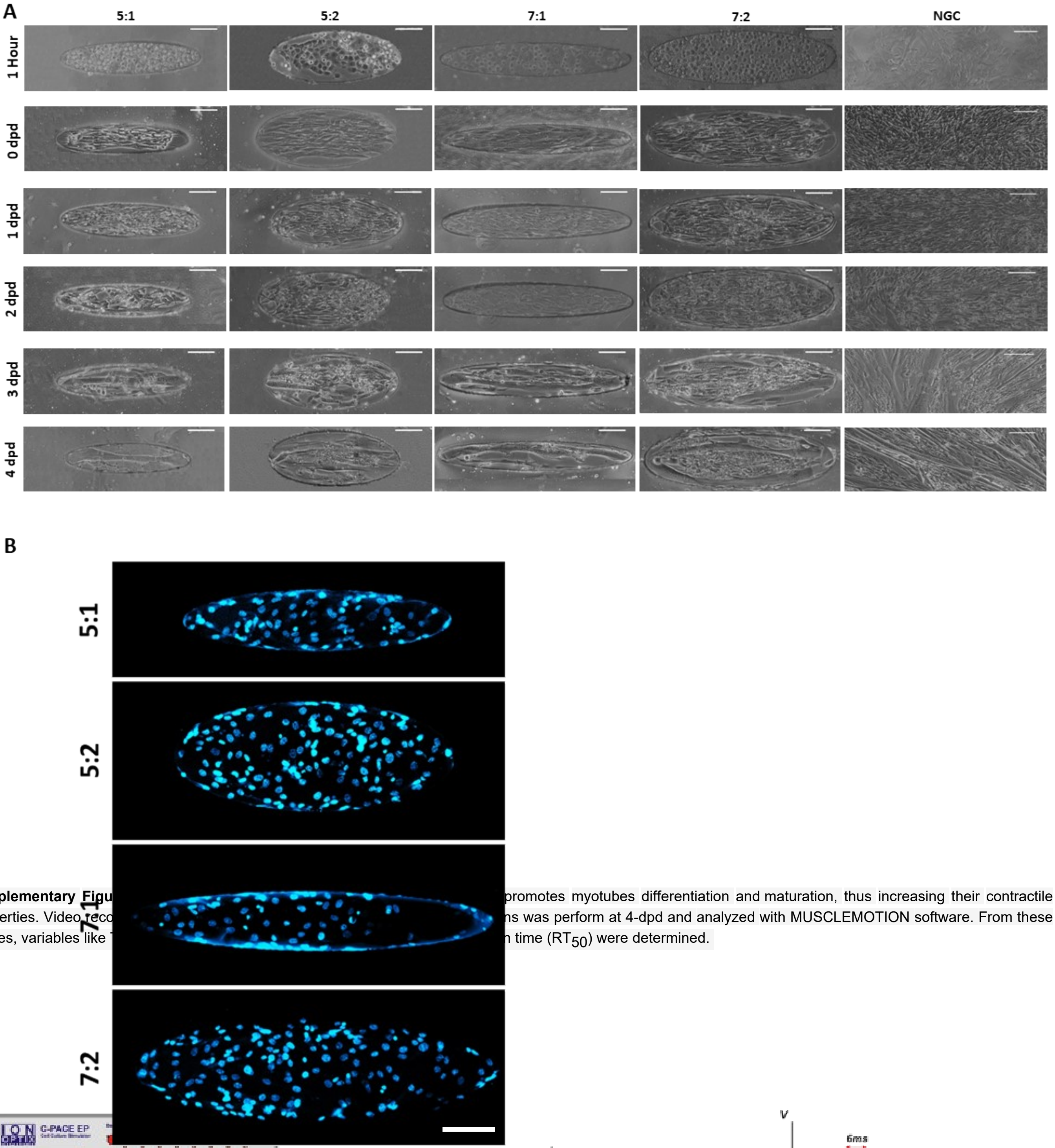
µGroove	µGroove /well	Height (80 µm)	Length (µm)	Wide (µm)	Ratio	Area (mm^2)	Seeding Area (mm^2)
5:1	263	75.64 ± 7.88	506.6 ± 9.00	119.1 ± 9.70	0.2	0.039	10.25
5:2	211	75.68 ± 5.09	507 ± 11.06	212.3 ± 10.29	0.4	0.078	16.45
7:1	257	78.08 ± 6.17	698.6 ± 16.19	115.1 ± 14.54	0.142	0.054	13.87
7:2	161	77.12 ± 3.07	705.1 ± 15.37	211.4 ± 8.981	0.285	0.109	17.54



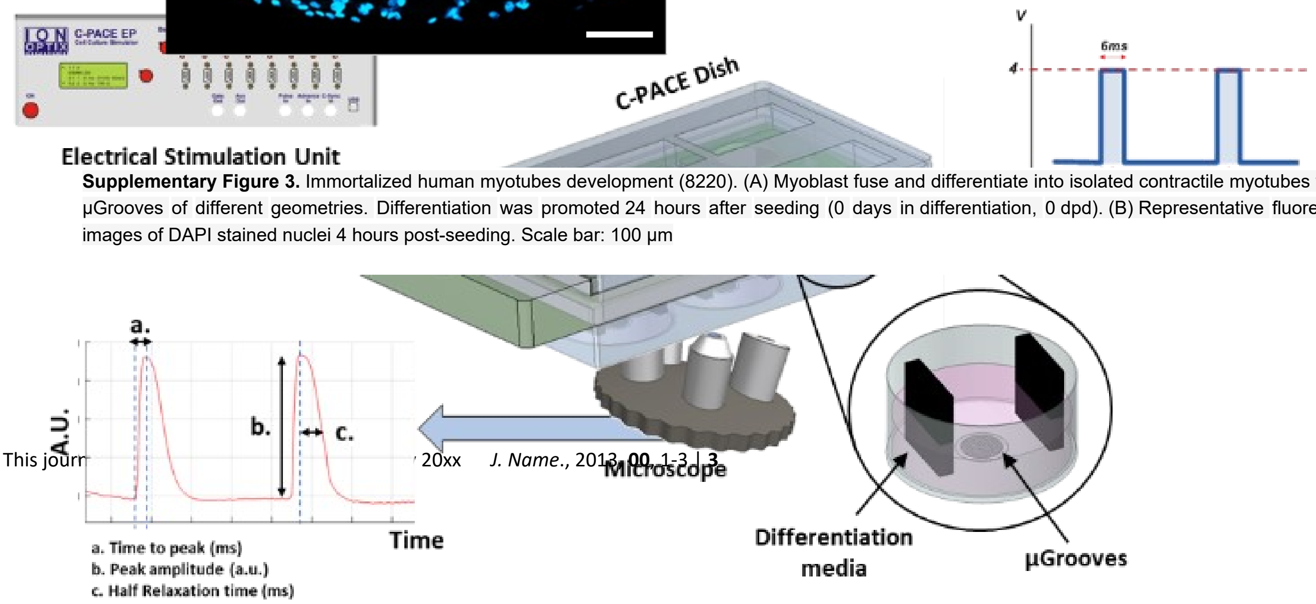
Supplementary Figure 1. Characterization of SU-8 masters and µGroove substrates. **(A)** Experimental dimensions of 5:1, 5:2, 7:1 and 7:2 µGroove geometries. For Si masters, five pictures were taken along the samples with the profilometer to consider possible inhomogeneities inherent to the fabrication process. Values are expressed as mean ± SD. **(B)** Representative images of SU-8 masters characterized with an optical profilometer. **(C)** Representative brightfield images of µGroove structures (5:2, left panel). In some cases, delamination artifacts were observed causing shape distortion or partially closed structures (right panel). Scale bar: 100 µm.

MEDIA	COMPONENTS	REF	BRAND
Growth Media	Skeletal muscle cell growh medium + Supplemented mix	PB-MH-272-0090	PELObiotech
	10% Fetal Bovine serum (FBS)	10270-106	Gibco
	50 µg/ml Gentamicin	15750037	Gibco
	1X Glutamax	35050038	Gibco
Basic Differentiation Media (bDM)	DMEM	41966029	Gibco
	100 µg/ml Apotransferine	T1147	SIGMA
	50 µg/ml Gentamicin	15750037	SIGMA
	10 µg/ml Insulin	I2643	SIGMA
Complete Differentiation Media (cDM)	Newobasal medium	10888022	Gibco
	1X B27 Supplement	17504044	Gibco
	20 ng/ml Brain Derived Neurotrophic Factor (BDNF)	450-02	PEPROTECH
	5 ng/ml Ciliary Neutrophic Factor CNTF	450-13	PEPROTECH
	50 µg /ml Gentamicin	15750037	GIBCO
	1X Glutamax	35050038	Gibco
	10 ng/ml Insulin-like growth Factor 1 IGF-1	I3769	SIGMA
	4 µg /ml Laminin	L2020	SIGMA
	20 ng/ml Neurotrophin 3 NT-3	450-03	PEPROTECH
	50 ng/ml Recombinant Human Sonic Hedgehog (Shh)	100-45	PEPROTECH
	100 ng/ml Recombinant Rat Agrin Protein	550-AG	R&D SYSTEMS

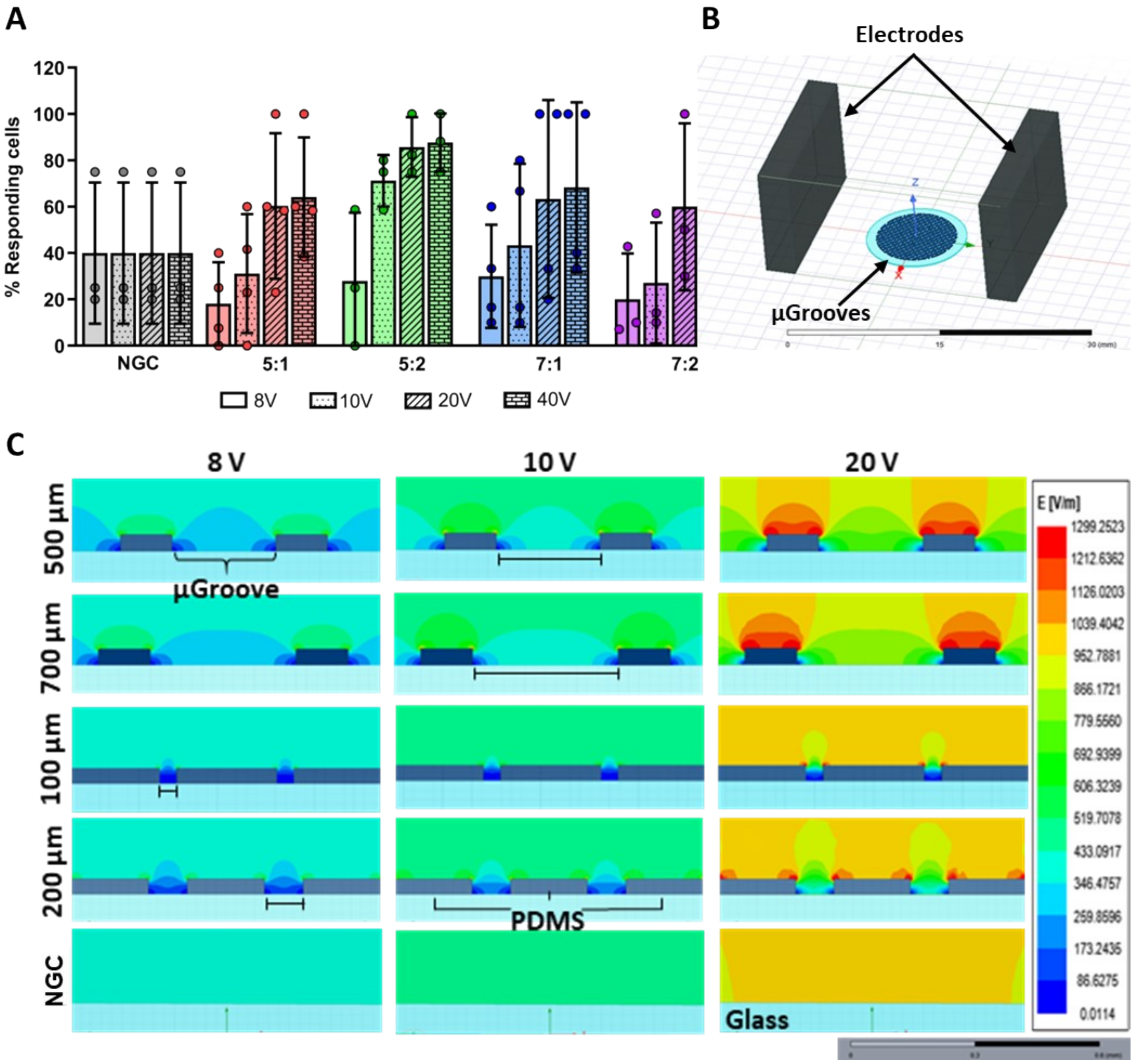
Supplementary Figure 2. Human immortalized skeletal muscle media components.



Supplementary Figu... promotes myotubes differentiation and maturation, thus increasing their contractile properties. Video reco... ns was perform at 4-dpd and analyzed with MUSCLEMOTION software. From these curves, variables like T... n time (RT_{50}) were determined.



Supplementary Figure 3. Immortalized human myotubes development (8220). (A) Myoblast fuse and differentiate into isolated contractile myotubes within µGrooves of different geometries. Differentiation was promoted 24 hours after seeding (0 days in differentiation, 0 dpd). (B) Representative fluorescent images of DAPI stained nuclei 4 hours post-seeding. Scale bar: 100 µm



Supplementary Figure 5. Myotubes Excitability. **(A)** Myotubes were electrical stimulated at increasing voltages (4-40 V). Random fields have been selected in the case of the controls and full grooves in the case of the different geometries. We calculated the % of responding myotubes under electrical stimulation as the ratio of those contracting to those not contracting in the selected field. All myotubes grown in the NGC responded at 8 V while for the ones grown within µGrooves, this response was highly dependent of voltage, increasing this parameter increase the number of myotubes that contract. **(B)** µGrooves were simulated in Ansys Electronics software to study the electric field behaviour in the µGrooves. **(C)** Electric field simulation for 8, 10 and 20 V in µGrooves and NGC. Voltages of 4 and 40 were not analyzed because, for the first case, we obtained no response in either µGrooves or NGC, and for the second case, all myotubes responded to this voltage. Width and length are displayed to check both orientations.

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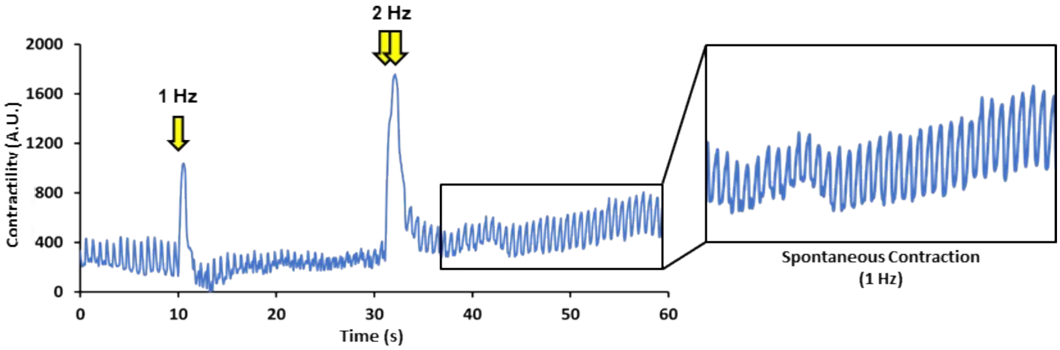


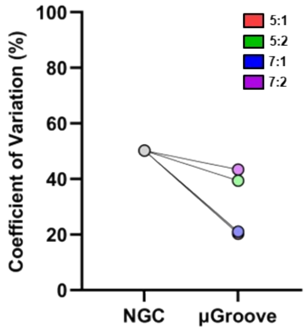
Supplementary Figure 6. Spontaneous contraction was observed after chronical stimulation with a frequency of approximately 1 Hz. In some cases, these spontaneous contractions were observed while evaluating the myotubes under increasing frequencies (yellow arrows).

A

Myotube width (µm)		
	µGroove	CV%
5:1	21.1 ± 4.27 (n=5)	20.3
5:2	14.3 ± 5.63* (n=7)	39.4
7:1	19.2 ± 4.05 (n=10)	21
7:2	23 ± 9.98 (n=15)	43.4
NGC	23.5 ± 11.8 (n=78)	50.2

Kruskal-Wallis test followed by Dunn's post-hoc (5:2 vs NGC, *p<0.05)

B



C

	Contractility (A.U.)					
	Twitch (0.1 Hz)	**CV%**	**Tetanus (10 Hz)**	**CV%**	**Tetanic-to-twitch Ratio**	**CV%**
5:1 (n=11)	393 ±186###	47.4	810 ± 374	46.2	2.22 ± 0.957	43
5:2 (n=17)	507 ±289###	56.9	794 ± 455	57.3	1.76 ± 0.897	51.1
7:1 (n=7)	577 ±299#	51.8	1332 ± 519	38.9	2.82 ± 1.36	48.4
7:2 (n=10)	764 ± 419	54.9	993 ± 373	37.6	1.54 ± 0.755	48.9
NGC (n=5)	651 ± 702	108	915 ± 538	58.8	2.28 ± 1.55	68.1

	Twitch kinetics (ms)			
	TTP	**CV%**	**RT**	**CV%**
5:1 (n=11)	287 ± 51.9	18.1	389 ± 70.1	18
5:2 (n=17)	403 ± 159	39.4	485 ± 213	44
7:1 (n=7)	304 ± 68.6	22.6	360 ± 61	17
7:2 (n=10)	302 ± 139	46.2	351 ± 131	37.2
NGC (n=5)	381 ± 49.1	12.9	446 ± 89	20

D

Resting cytosolic Ca^{2+}(nM)		
	µGroove	CV%
5:1	32.9 ± 8.21** (n=5)	25
5:2	62.2 ± 17.5 (n=7)	28.2
7:1	69.6 ± 21.9 (n=10)	31.4
7:2	39.8 ± 19.9 (n=15)	49.9
NGC	57 ± 31.5 (n=15)	55.2

Welch ANOVA followed by Dunnett's post-hoc test were used for statistical analysis. (5:1 vs NGC, **p < 0.01)

E

	Cytosolic calcium fluxes (ΔF/F)					
	Twitch (0.1 Hz)	**CV%**	**Tetanus (10 Hz)**	**CV%**	**Tetanic-to-twitch Ratio**	**CV%**
5:1 (n=5)	0.045 ± 0.016	35.3	0.067 ± 0.016	24.1	1.69 ± 0.90	53.4
5:2 (n=4)	0.160 ± 0.050*	31.8	0.228 ± 0.103	45.3	1.41 ± 0.41	29.3
7:1 (n=11)	0.085 ± 0.054##	63	0.223 ± 0.140*	63	2.70 ± 0.43	16.1
7:2 (n=7)	0.164 ± 0.162	98.7	0.274 ± 0.320	117	1.78 ± 1.07	60.5
NGC (n=25)	0.072 ± 0.079##	109	0.115 ± 0.143	125	2.07 ± 5.20	94.7

Kruskal-Wallis test and Dunn's post-hoc test were used for statistical analysis. *p < 0.05. Paired t-test was used to analyze difference between twitch and tetanus contractions for 5:1, 5:2, 7:1 geometries. ###p < 0.001. Wilcoxon matched-pairs signed rank test was used for 7:2 µGrooves and NGC. ##p < 0.01

Supplementary Figure 7. Coefficient of variation percentage (CV%) of µGrooved myotubes and non-grooved controls (NGC) for different assays. **(A)** Myotube width (µm). (B) Coefficient of variation (%CV) of myotube width datasets. The %CV decreased by 59.56%, 27%, 59.76% and 13.54% for 5:1, 5:2, 7:1 and 7:2 µGrooves, respectively, compared to NGC. Dots represent values from different µGroove geometries. (C) Twitch and tetanic contractility measured by peak amplitude (A.U., arbitrary units; left panel), and twitch kinetics for time to peak (TTP) and half-relaxation time (RT_{50}; right panel) (D) Resting cytosolic Ca^{2+} (nM). (E) Cytosolic calcium fluxes in response to twitch and tetanic stimuli. Statistical analyses performed are detailed below each table.

Supplementary Video 1 Spontaneous Contractions in μGrooves substrates.

