Mechanical confinement regulates cartilage matrix formation by chondrocytes

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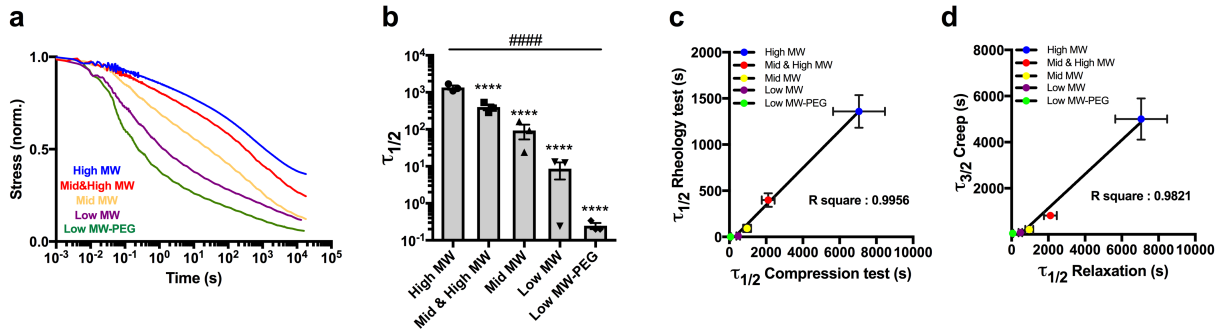
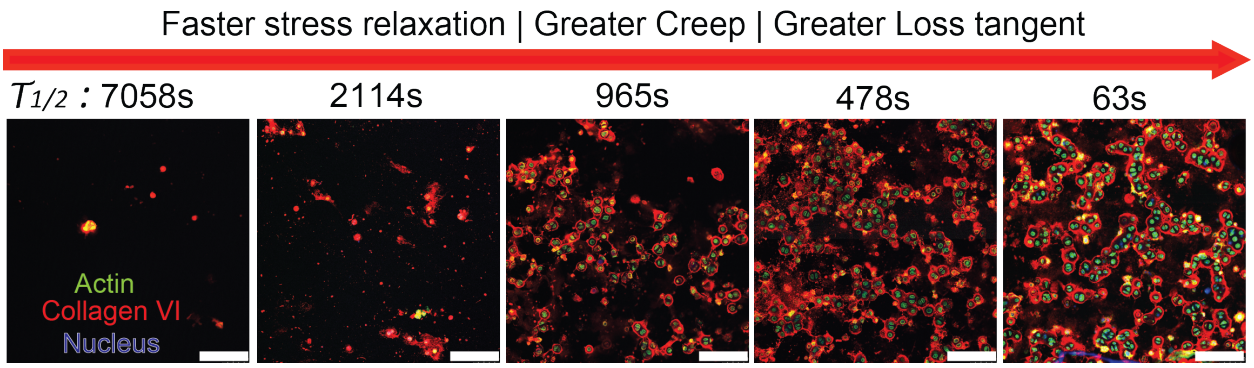
Supplementary Information

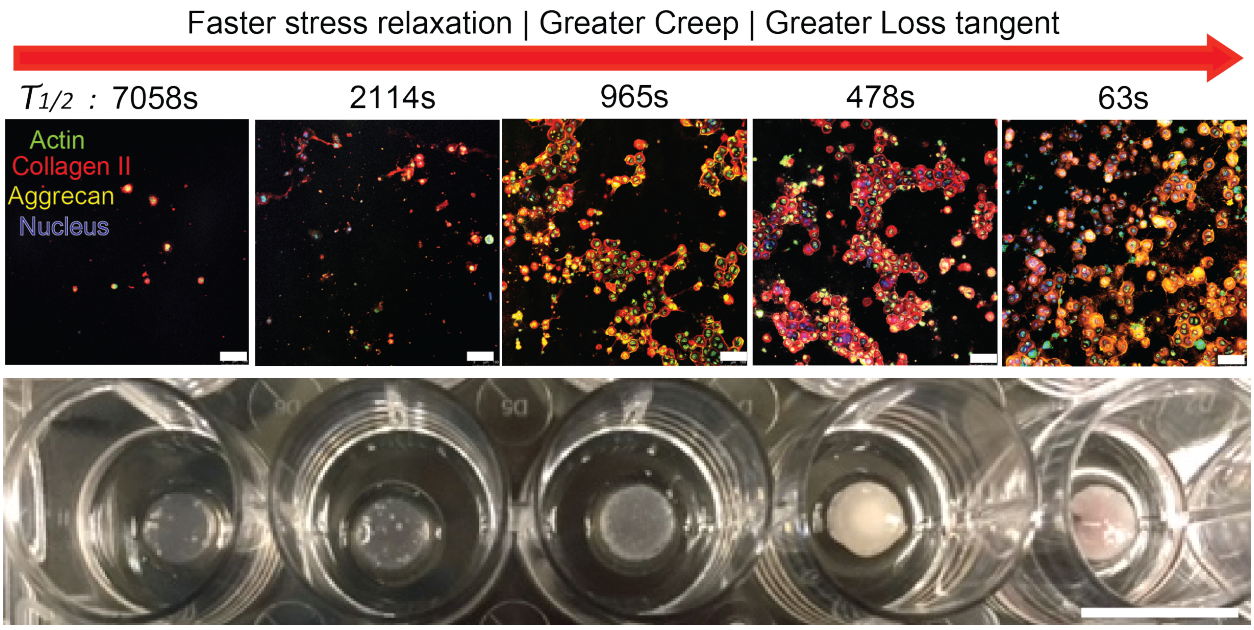
Supplementary Figures and Legends



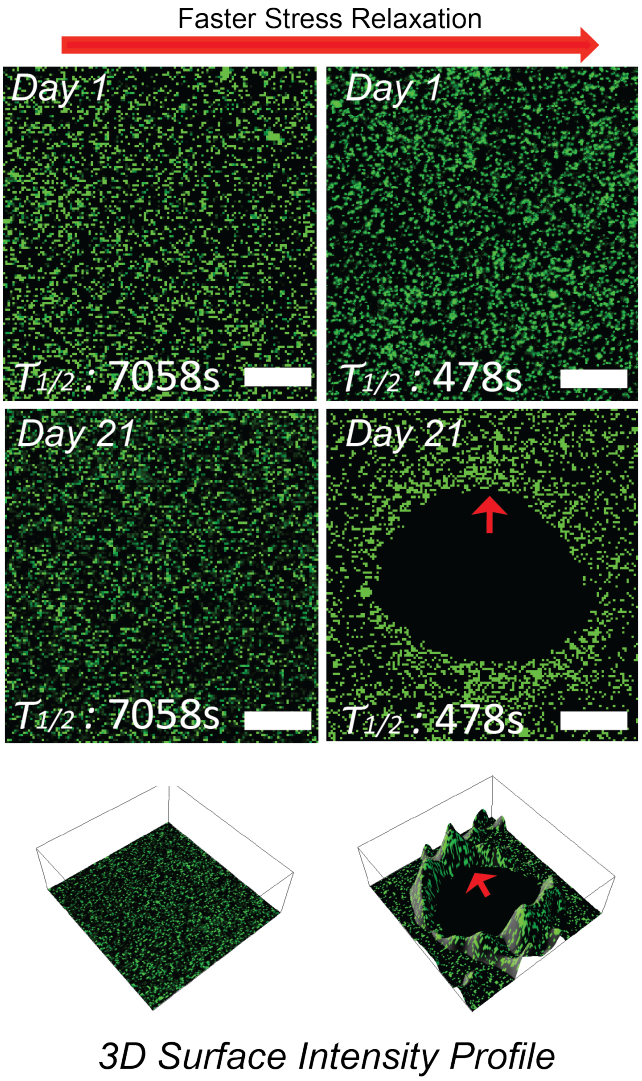
Supplementary Figure 1 | Rheology for stress relaxation of alginate hydrogels and a linear correlation between the timescale for creep and the timescale for stress relaxation. a, Shear stress relaxation profile of alginate hydrogels composed of alginate of varying molecular weights, or modified with a short PEG spacer, conducted in a rheometer. For all experiments, a 15% shear strain was applied. **b**, Quantification of the time scale of stress relaxation for the different alginate hydrogels (n=3 replicates per conditions, #### $p < 0.0001$ by Spearman's rank correlation and ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared to high MW alginate condition by one-way ANOVA). **c**, Scatter plots of the relaxation times ($\tau_{1/2}$) from compression or shear. Linear regression analysis indicated a linear correlation between two groups of relaxation time (R square = 0.9956). **d**, Scatter plots between the relaxation times ($\tau_{1/2}$) from compression and the creep times ($\tau_{3/2}$) from shear. Linear regression analysis indicated a linear correlation between two groups of timescales (R square = 0.9821). Data are shown as mean ± s.e.m.



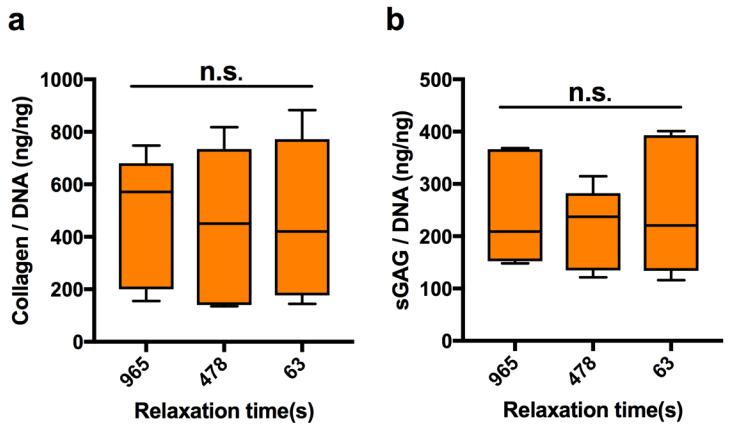
Supplementary Figure 2 | Pericellular matrix deposition in 3kPa hydrogels. Representative stainings of type VI collagen produced by chondrocytes cultured in 3kPa hydrogels for 21 days in the indicated conditions. Scale bar, 200 μm.



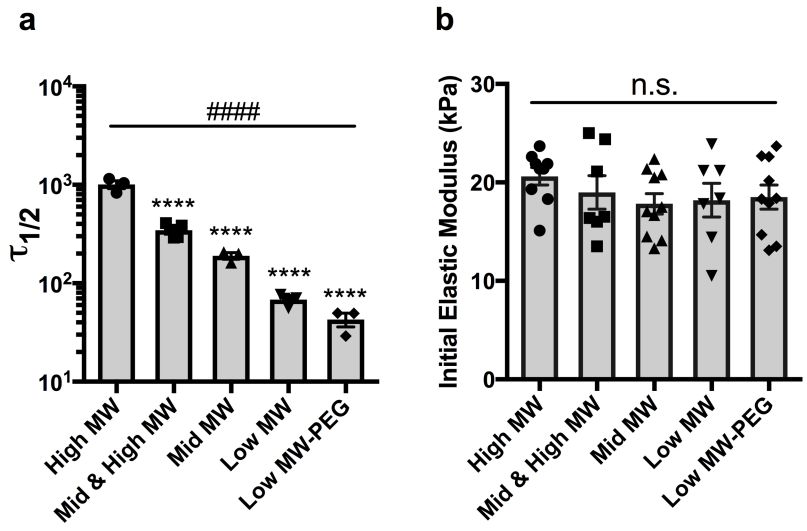
Supplementary Figure 3 | Cartilage matrix deposition and macroscopic appearance of hydrogels containing chondrocytes after 21 days of culture. a, Immunohistochemical stains of chondrocytes cultured in 3kPa hydrogels with varying levels of stress relaxation for 21 days. Scale bar, 100 μm. **b**, Increased opacity was observed in hydrogels with faster stress relaxation at day 21, and the size of the constructs were found to be similar. Scale bar,12 mm.



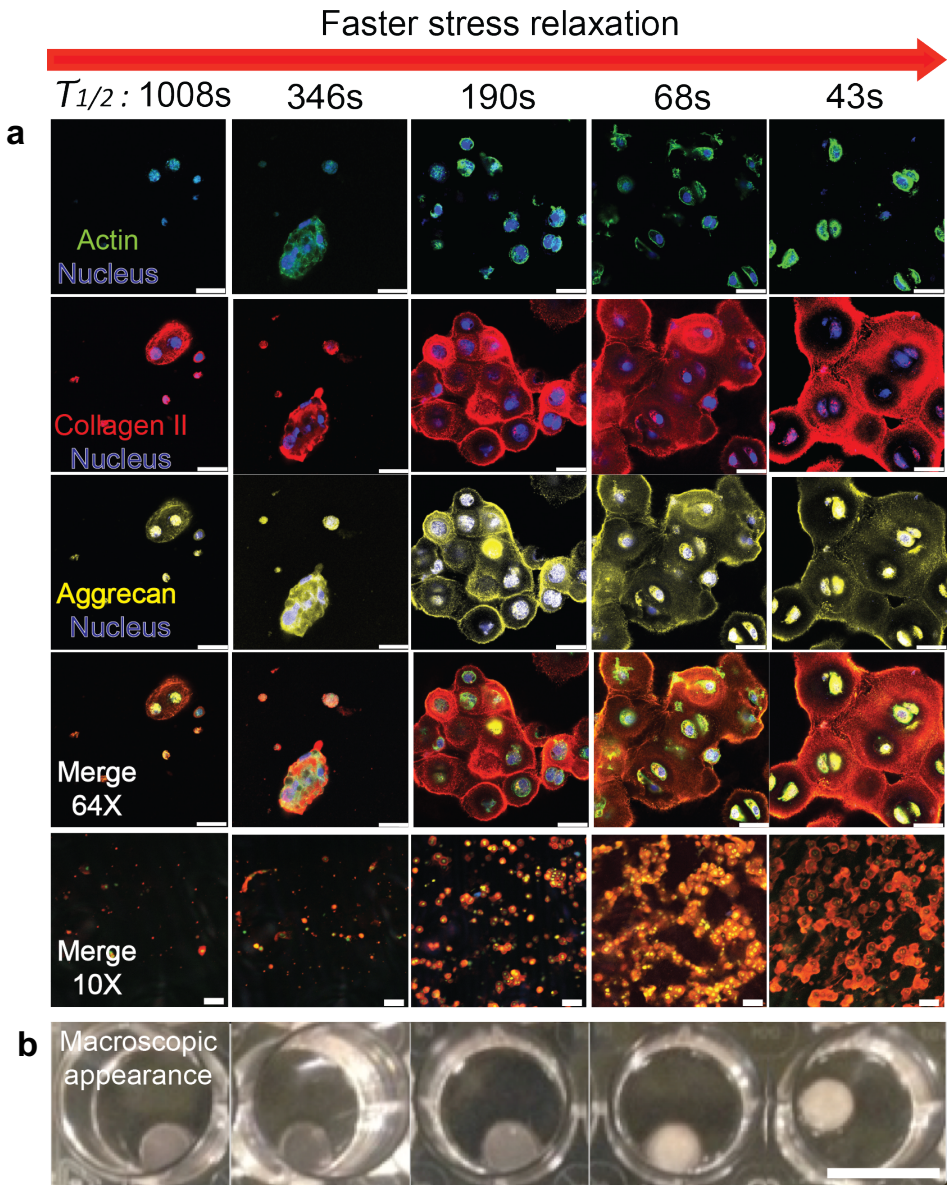
Supplementary Figure 4 | Displacement of hydrogels around cells in fast relaxing gels after 21 days of culture. Representative images of fluorescent microbeads encapsulated in 3kPa hydrogels in indicated conditions for 1 day and 21 days. The distribution of beads was relatively uniform at 1 day of culture, and after 21 days in the slow relaxing gels. A higher density of beads was observed in areas immediately surrounding the regions that presumably contain chondrocytes and cartilage matrix in fast relaxing gels, indicating matrix displacement. Green color represents microbeads encapsulated in hydrogels. Scale bar, 50 μm.



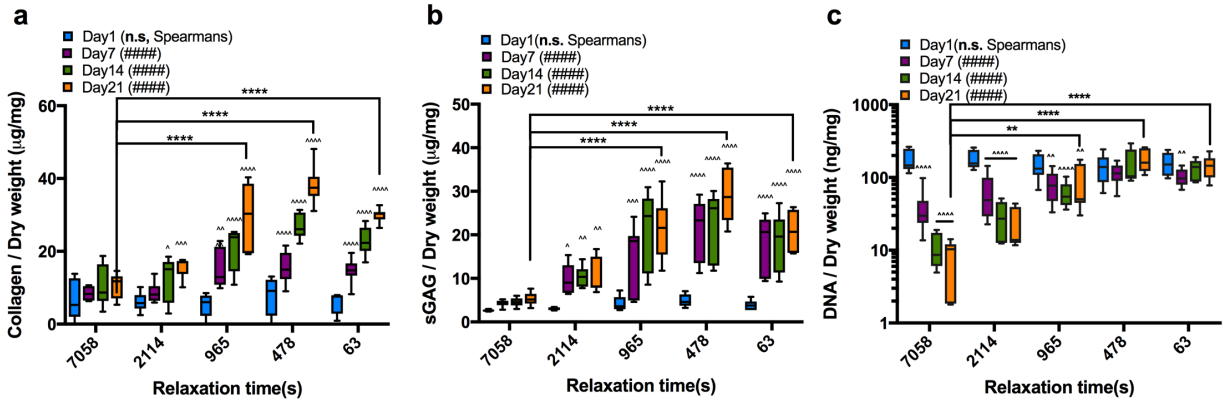
Supplementary Figure 5 | Amounts of cartilage matrix components normalized to DNA at day 21. Quantification of (a) collagen and (b) sGAG produced by chondrocytes cultured in 3kPa hydrogels with stress relaxation times ranging from $\tau_{1/2}$ = 965 seconds to 63 seconds for day 21. No significant differences among normalized amounts of both collagen and sGAG were measured (one-way ANOVA, $p > 0.9999$ for collagen, $p > 0.0883$ for sGAG). The box plots show 25/50/75th percentiles, whiskers show minimum/maximum, n=4 biological replicates per conditions.



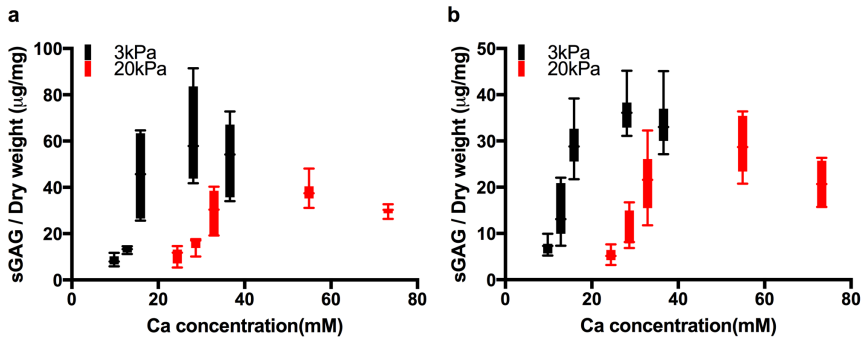
Supplementary Figure 6 | Modulation of stress relaxation rate in alginate hydrogels with an initial elastic modulus of 20kPa a, Quantified time scales of stress relaxation for stiff alginate hydrogels (n=3 replicates per conditions, #### $p < 0.0001$ by Spearman's rank correlation and **** $p < 0.0001$ compared to high MW alginate condition by one-way ANOVA). **b**, Initial elastic moduli of the stiff alginate hydrogels are similar for all formulations (n=7 replicates per conditions, $p > 0.3130$ compared to high MW alginate condition by one-way ANOVA). Data are shown as mean ± s.e.m.



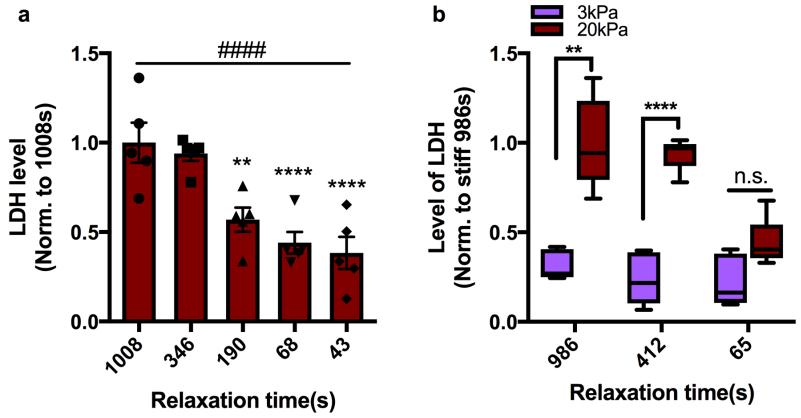
Supplementary Figure 7 | Cartilage matrix deposition by chondrocytes cultured in 20 kPa hydrogels a, Immunohistochemical stains of chondrocytes cultured in 20 kPa hydrogels with varying levels of stress relaxation for 21 days. Scale bar is 25 μm for 64X images and 100 μm for 10X images. **b,** Increased opacity was observed in 20kPa hydrogels with faster stress relaxation at day 21, and the size of the constructs were found to be similar. Scale bar, 12 mm.



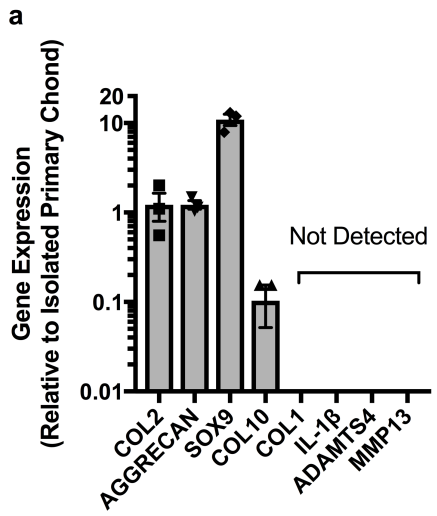
Supplementary Figure 8 | Amounts of cartilage matrix components and DNA contained in 20 kPa hydrogels containing chondrocytes after 3 weeks of culture a-c, Quantification of (a) collagen, (b) sGAG and (c) DNA from constructs consisting of chondrocytes cultured in 20 kPa hydrogels with varying stress relaxation for 21 days. #### located beside each legend indicates $p < 0.0001$(Spearman's rank correlation). ^^, ^^^, and ^^^^ indicate statistically significant differences when compared to value at Day 1 with p <0.01, 0.001, and 0.0001 respectively (two-way ANOVA). **, *** and **** indicate statistically significant differences when compared to slow relaxing gel condition ($\tau_{1/2}$ = 1008s) with $p < 0.01$, 0.001 and 0.0001 respectively (two-way ANOVA). The box plots show 25/50/75th percentiles, whiskers show minimum/maximum, n=4 biological replicates per conditions.



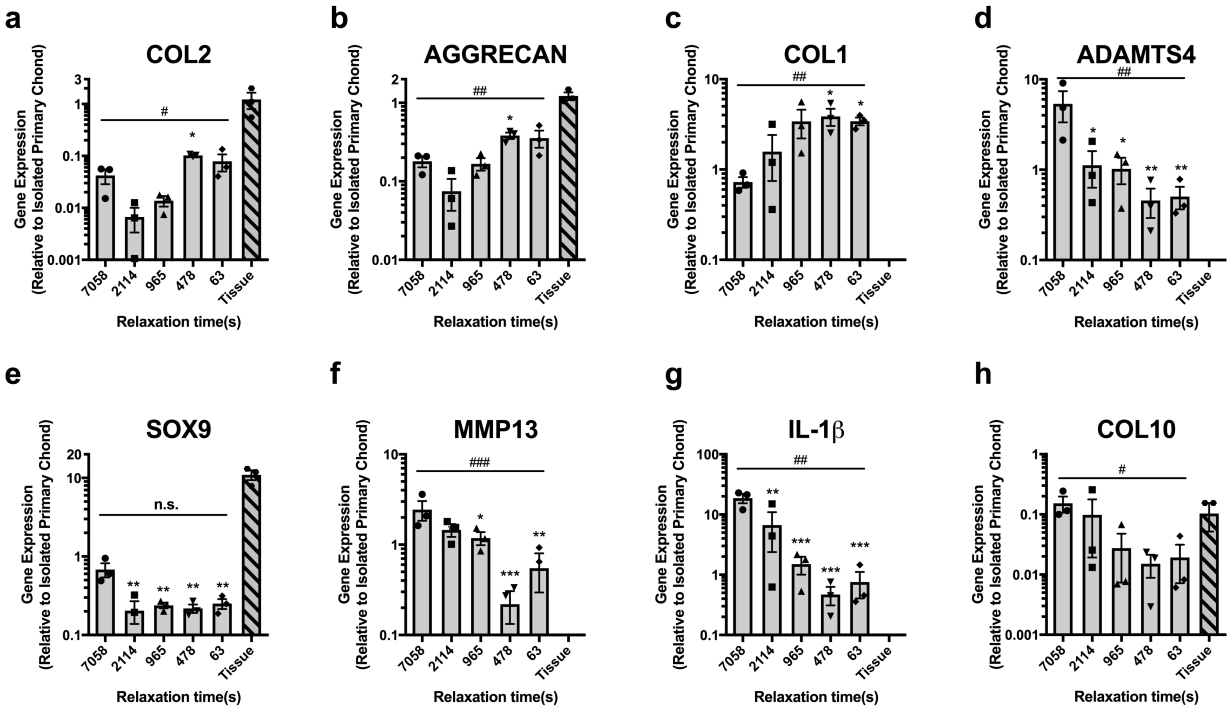
Supplementary Figure 9 | Amounts of cartilage matrix deposition are not correlated with calcium crosslinker concentration. Quantification of (a) collagen and (b) sGAG from constructs containing chondrocytes cultured in 3kPa and 20 kPa hydrogels for 21 days as a function of the concentration of calcium used to crosslink the matrix. There is no correlation between calcium concentration and the amounts of cartilage matrix components produced by chondrocytes (Spearman rank correlation, $p > 0.233$ for collagen, $p > 0.0833$ for sGAG). The box plots show 25/50/75th percentiles, whiskers show minimum/maximum, n=4 biological replicates per conditions.



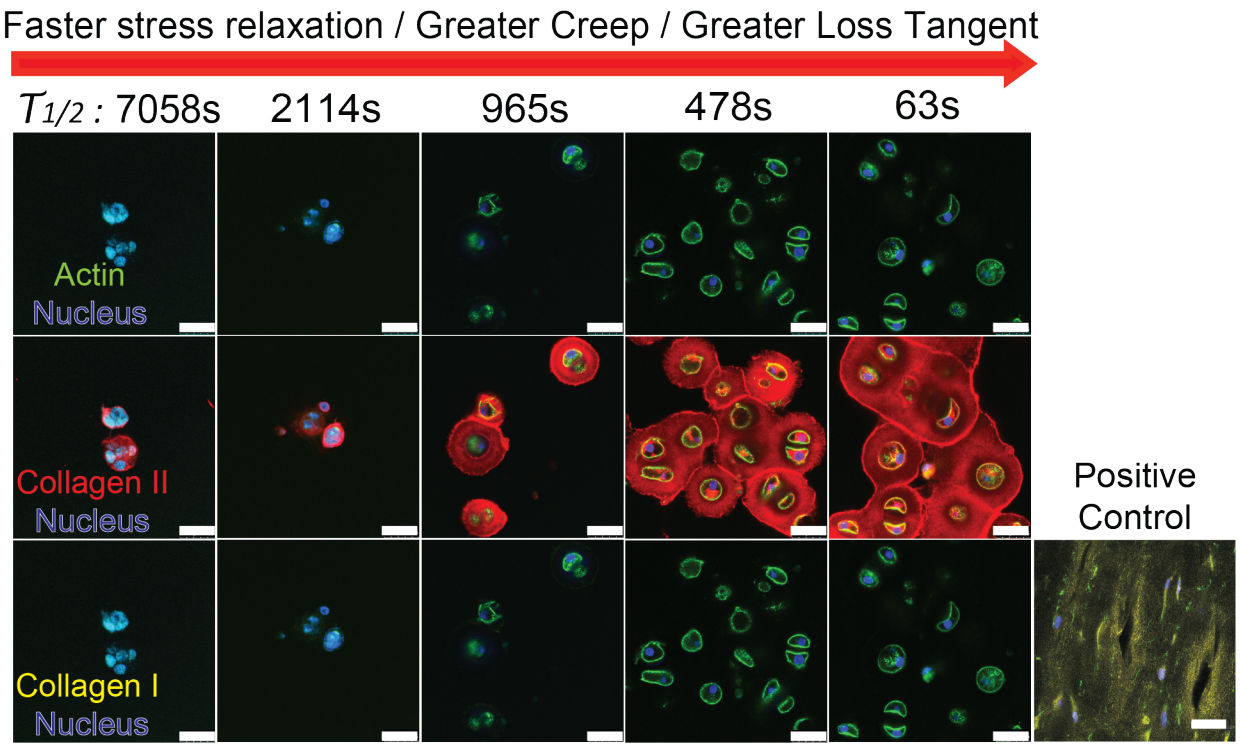
Supplementary Figure 10 | Cell death levels are greater in stiffer gels. a, Quantification of LDH levels from constructs consisting of chondrocytes cultured in 20 kPa hydrogels varying levels of stress relaxation for 7 days. Values are normalized by the value in the slowest relaxing gel condition ($\tau_{1/2}$ = 1008s). (#### $p < 0.0001$ by Spearman's rank correlation and ** $p < 0.01$, **** $p < 0.0001$ compared to slow relaxing gel condition ($\tau_{1/2}$ = 1008s) by one-way ANOVA, mean ± s.e.m.). **b**, Comparison of LDH levels for chondrocytes encapsulated in stiff or soft alginate gels. (**$p < 0.01$and**** $p < 0.0001$ by student's t-test). The box plots show 25/50/75th percentiles, whiskers show minimum/maximum, n=5 replicates per conditions.



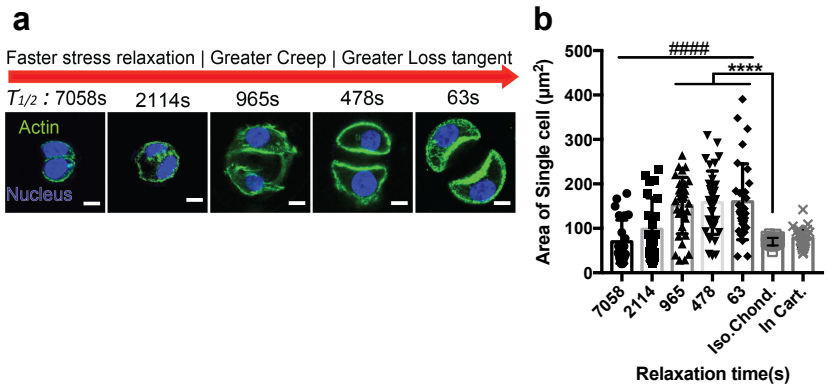
Supplementary Figure 11 | Gene expression of chondrocytes harvested from bovine articular cartilage. Quantification of relative gene expression of type II collagen (COL2), aggrecan (AGGRECAN), Sox9 (SOX9), type X collagen (COL10), type I collagen (COL1), IL-1β, an aggrecanase (ADAMTS4), and a collagenase (MMP13) from chondrocytes extracted from articular cartilage. Values are normalized by the value measured in isolated primary chondrocytes. Data are shown as mean ± s.e.m, n=3 replicates per conditions.



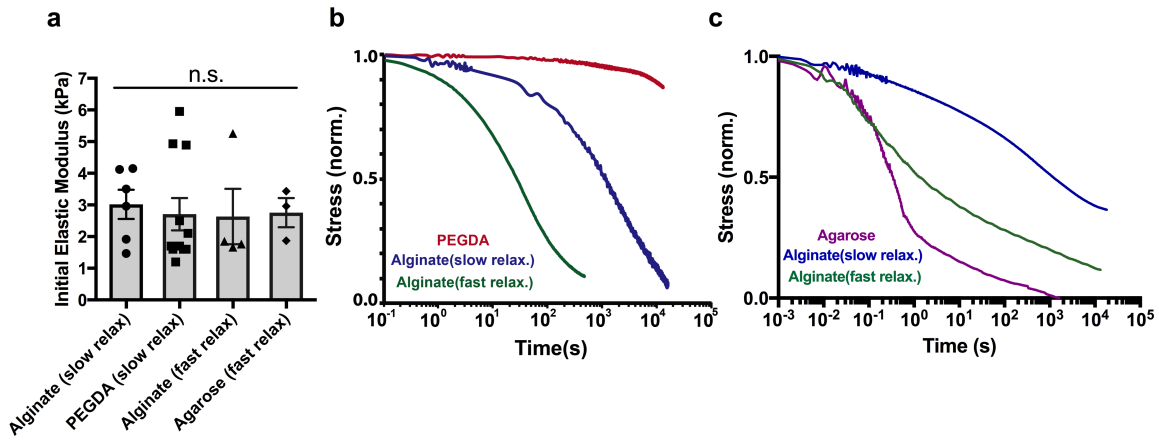
Supplementary Figure 12 | Gene expression in chondrocytes cultured in 3 kPa hydrogels for 21 days a-h, Quantification of relative gene expression of (a) type 1 collagen (COL1), (b) an aggrecanase (ADAMTS4), (c) a collagenase (MMP13), (d) Sox9 (SOX9), (e) IL-1β and (f) type X collagen (COL10). Values are normalized by the value measured in isolated primary chondrocytes. #, ## and ### on the top indicate p <0.05, 0.01, and 0.001 respectively (Spearman's rank correlation). *, **, ***. and **** indicate statistically significant differences when compared to the slow relaxing gel condition ($\tau_{1/2}$ = 1008s) with $p < 0.01, 0.001$ and 0.0001 respectively (one-way ANOVA). All data are shown as mean ± s.e.m., n=3 replicates per conditions.



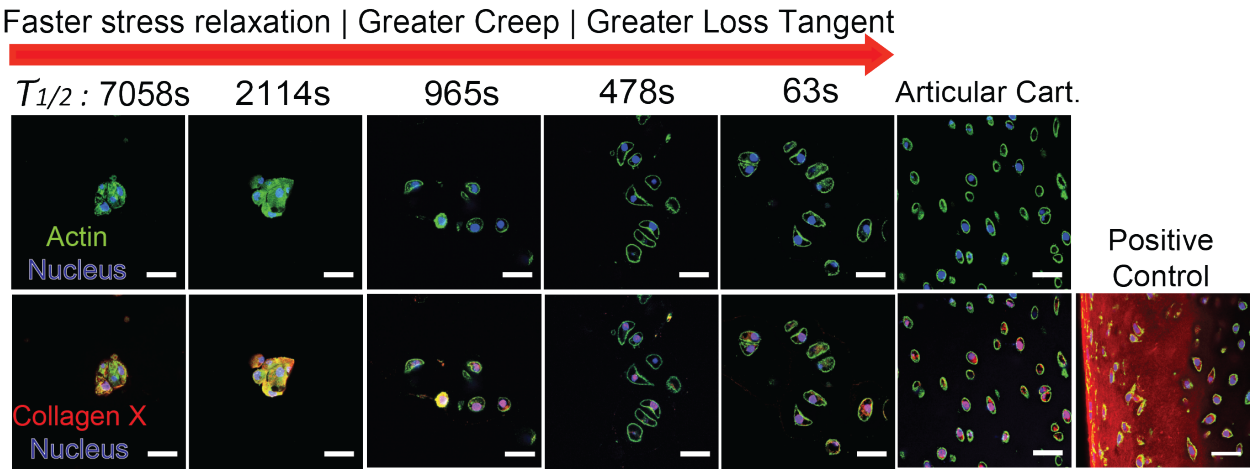
Supplementary Figure 13 | Type I collagen and type II collagen deposition by chondrocytes cultured in 3 kPa gels Representative stainings of collagen produced by chondrocytes cultured in 3kPa hydrogels for 21 days with the indicated timescale of stress relaxation, and in bovine fibrocartilage as a positive control. No staining of type I collagen is observed. Scale bar, 25 μm.



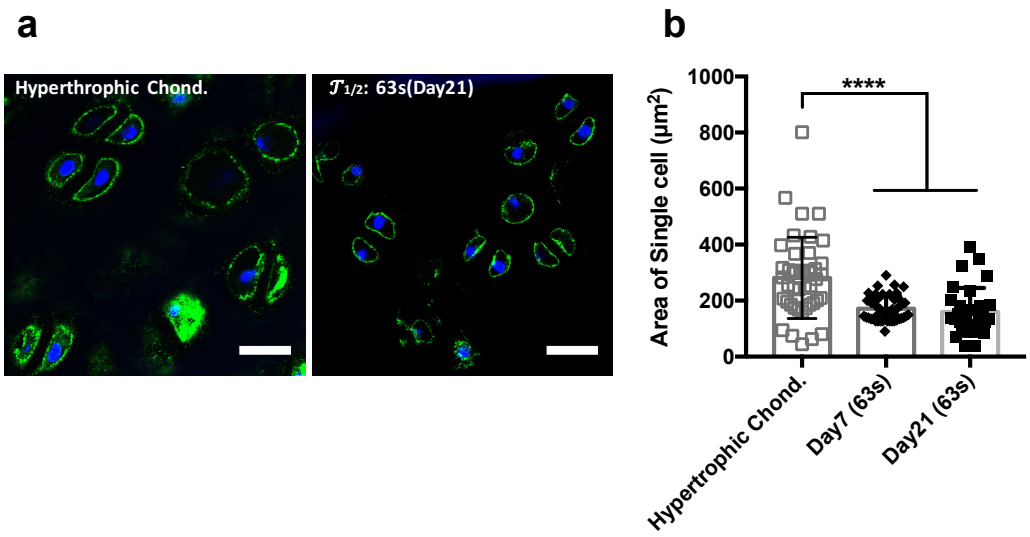
Supplementary Figure 14 | Size differences of chondrocytes cultured in 3 kPa alginate hydrogels for 21 days a, Representative immunofluorescence images with staining for actin (green) and nucleus (blue) for chondrocytes cultured in the indicated conditions for three weeks. Scale bar, 5μm. **b**, Quantification of single cell size for chondrocytes in hydrogels indicated conditions after three weeks of culture ($n > 50$ cells per conditions). The size of isolated primary chondrocytes (Iso. Chond.) and the size of chondrocytes in articular cartilage (In Cart.) are included for comparison. #### located on the top indicates $p < 0.0001$ (Spearman's rank correlation). **** indicate statistically significant differences when compared to control (Iso. Chond.) with $p < 0.0001$ respectively (one-way ANOVA).



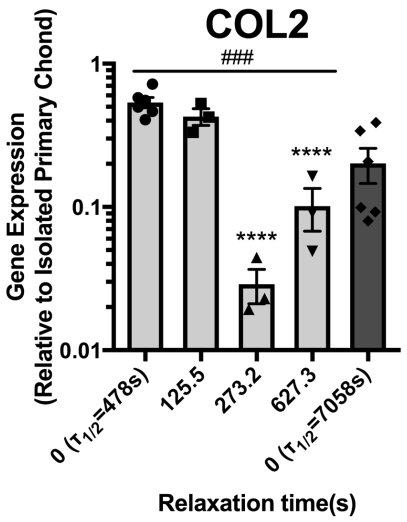
Supplementary Figure 15 | Initial elastic modulus and stress relaxation of PEG and agarose hydrogels. **a**, Initial elastic modulus of the PEG and agarose hydrogels measured using compression. No significant differences between elastic moduli were detected (one-way ANOVA, $p = 0.9211$). Data are shown as mean ± s.e.m., n>3 replicates per conditions. **b**, Stress relaxation profile of PEG hydrogels. Stress relaxation of fast relaxing (green, $\tau_{1/2}$ = 478s) and slow relaxing (blue, $\tau_{1/2}$ = 7058s) alginate gels are shown for comparison. **c**, Shear stress relaxation profile of agarose hydrogels conducted in a rheometer with a 15% shear strain. Stress relaxation of fast relaxing and slow relaxing alginate gels are shown for comparison.



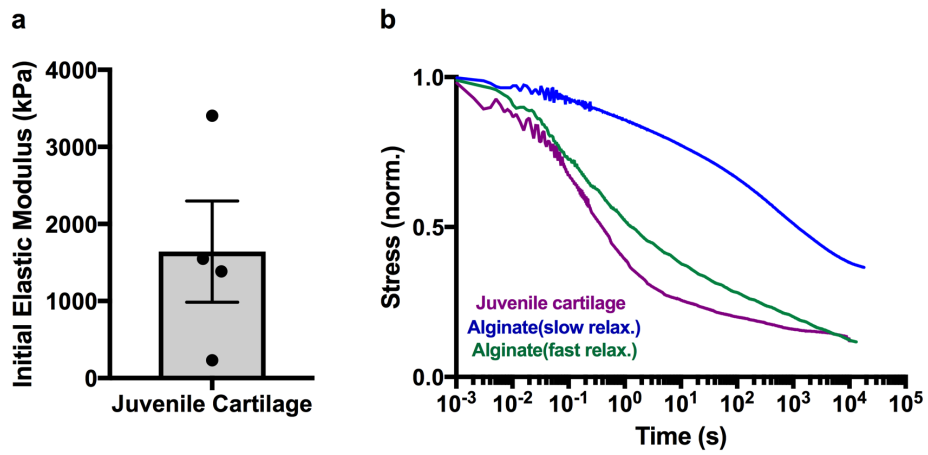
Supplementary Figure 16 | Type X collagen deposition by chondrocytes cultured in 3 kPa gels. Representative stainings of type X collagen produced by chondrocytes cultured in 3kPa hydrogels for 21 days with the indicated timescale of stress relaxation. No staining of type X collagen is observed for cells in the hydrogels. Staining of articular cartilage (Articular Cart.) is shown for comparison. Scale bar, 25 μm.



Supplementary Figure 17 | Size differences between hypertrophic chondrocytes in the deep zone of bovine articular cartilage and chondrocytes cultured in the fast relaxing hydrogel for 21 days a, Representative immunofluorescence images with staining for actin (green) and nucleus (blue) for chondrocytes in the indicated conditions. Scale bar, 25μm. **b**, Quantification of single cell size of hypertrophic chondrocytes in the deep zone in bovine articular cartilage and for chondrocytes in hydrogels of indicated conditions. **** indicates statistically significant differences when compared to the case of hypertrophic chondrocytes with $p < 0.0001$ respectively (one-way ANOVA).



Supplementary Figure 18 | Gene expression of type II collagen impacted by osmotic pressure. Quantification of relative gene expressions of type II collagen to that of isolated primary chondrocytes after 7 days of culture in the indicated conditions. Gene expression of type II collagen decreased significantly with higher osmotic pressure (### p <0.001 by Spearman's rank correlation and *** $p < 0.001$, **** $p < 0.0001$ compared to control ($\tau_{1/2}$ = 478s) by one-way ANOVA). Data are shown as mean ± s.e.m., n=3 replicates per conditions.



Supplementary Figure 19 | Initial elastic modulus and stress relaxation of juvenile cartilage. **a**, Initial elastic modulus of the juvenile cartilage measured using unconfined compression. Data are shown as mean ± s.e.m., n=4 replicates. **b**, Shear stress relaxation profile of juvenile cartilage conducted in a rheometer with a 15% shear strain. Stress relaxation of fast relaxing and slow relaxing alginate gels are shown for comparison. Juvenile cartilage exhibits fast stress relaxation.

Supplementary video 1 | Brightfield time-lapse videos of chondrocytes for 44 hours following encapsulation in fast relaxing hydrogel. Chondrocyte exhibits volume expansion.

Supplementary video 2 | Brightfield time-lapse videos of chondrocytes for 44 hours following encapsulation in slow relaxing hydrogel. Chondrocyte does not expand its volume.

Supplementary Table 1 | List of all hydrogel compositions used in study.

Description	Avg. MW of alginate (kDa)	Cross-linker conc. (mM)	Alginate conc. (wt/vol, %)	Initial elastic modulus (kPa, mean +/- s.d.)	Stress relaxation halftime, $\tau_{1/2}$ (s, mean +/- s.d.)
High MW low stiffness	280	9.8	2	3.0 +/- 1.1	7058 +/- 1416
Mid & High MW low stiffness	280 & 70	12.8	2	3.5 +/- 0.8	2114 +/- 348
Mid MW low stiffness	70	15.9	2	3.0 +/- 1.0	965 +/- 216
Low MW low stiffness	35	28.1	2	2.7 +/- 1.6	478 +/- 97
Low MW-5K PEG low stiffness	35	36.6	2	2.8 +/- 0.9	63 +/- 25
High MW high stiffness	280	24.4	2	18.5 +/- 3.8	1007 +/- 163
Mid & High MW high stiffness	280 & 70	28.7	2	18.2+/- 4.5	345 +/- 53
Mid MW high stiffness	70	32.9	2	17.8 +/- 3.2	189 +/- 25
Low MW high stiffness	35	54.9	2	19.0 +/- 4.5	68 +/- 8
Low MW-5K PEG high stiffness	35	73.2	2	19.9 +/- 2.5	42 +/- 12

Supplementary Table 2 | List of specific primers for real-time polymerase chain reaction.

Name	Forward/Reverse	T(℃)	Gen Bank No.	References
COL1A2 (COL1)	**F: 5' ACATTGGCCCAGTCTGTTTC 3'**	**55**	**NM_174520.2**	58
	R: 5' GGGAGGGGGAGTGAATTAAA 3'			
COL2A1 (COL2)	**F: 5' GTGGGGCAAGACTATGATCG 3'**	**55**	**NM_001113224**	58
	R: 5' TGCAATGGATTGTGTTGGTT 3'			
COL10A1 (COL10)	**F: 5' TTCTTCATCCCGTATGCCA 3'**	**53**	**NM_174634.1**	59
	R: 5' GGTGGACCAGGGATGCC 3'			
Aggrecan (AGGRECAN)	**F: 5' CACCACAGCAGGTGAACTAGA 3'**	**56**	**NM_173981.2**	58
	R: 5' GCTTGCTCCTCCACTAATGTC 3'			
Sox9 (SOX9)	**F: 5' ACGCGGCCCCAGGAGAAC 3'**	**60**	**XR_083993.1**	27
	R: 5' CGGATGCACACGGGGAACTT 3			
MMP13 (MMP13)	**F: 5' TTCTGGCACACGCTTTTCCTC 3'**	**58**	**NM_174389.2**	60
	R: 5' GGTTGGGGTCTTCATCTCCTG 3'			
ADAMTS4 (ADAMTS4)	**F: 5' TGGATCCTGAGGAGCCCTG 3'**	**59**	**NM_181667.1**	61
	R: 5' TGGCGGTCAGCATCATAGTC 3'			
Interleukin-1β (IL-1β)	**F: 5' GCTCTCCACCTCCTCTCACAG 3'**	**59**	**NM_174093.1**	61
	R: 5' TACATTCTTCCCTTCCCTTCT 3'			
Beta-actin (β actin)	**F: 5' GGTCATCACCATTGGCAATG 3'**	**54**	**NM_173979.3**	59
	R: 5' CCACAGGACTCCATGCCC 3'			

Supplementary Table 3 | Details for statistical analyses. F indicates F-values for ANOVA tests, T indicates t-values for t-tests, and df indicates degrees of freedom.

Figure	Analysis	One-tail or Two-tail	F (dfn, dfd) or T, df
Figure 1b	One-way ANOVA	Two-tail	F (4, 25) = 115.1
Figure 1c	One-way ANOVA	Two-tail	F (4, 15) =0.3048
Figure 1d	One-way ANOVA	Two-tail	F (4, 10) = 25.68
Figure 1f	One-way ANOVA	Two-tail	F (4, 10) = 18.89
Figure 1g	Student t-test	Two-tail	t=0.431 df=4
	Two-way ANOVA	Two-tail	Relax: F (4, 27) = 2.217 Time: F (1, 27) = 0.1105
Figure 1h	Student t-test	Two-tail	t=0.6109 df=4
	Two-way ANOVA	Two-tail	Relax: F (4, 30) = 0.799 Time: F (1, 30) = 0.2981
Figure 2c	One-way ANOVA	Two-tail	F (4, 25) = 45.55
Figure 2d	Two-way ANOVA	Two-tail	Relax: F (4, 117) = 48.42 Time: F (3, 117) = 77.57
Figure 2e	Two-way ANOVA	Two-tail	Relax: F (4, 201) = 141.9 Time: F (3, 201) = 193.4
Figure 2f	Student t-test	Two-tail	986s: t=4.801 df=10 412s: t=6.155 df=10 65s: t=2.235 df=11
Figure 2g	Student t-test	Two-tail	986s: t=13.55 df=19 412s: t=13.01 df=16 65s: t=1.955 df=19
Figure 3a	Two-way ANOVA	Two-tail	Relax: F (4, 204) = 29.44 Time: F (3, 204) = 33.05
Figure 3c	One-way ANOVA	Two-tail	F (4, 10) = 19.86
Figure 3d	One-way ANOVA	Two-tail	F (4, 22) = 7.361
Figure 3e	One-way ANOVA	Two-tail	F (4, 13) = 20.3
Figure 3f	One-way ANOVA	Two-tail	F (4, 13) = 8.849
Figure 3g	One-way ANOVA	Two-tail	F (4, 13) = 2.017
Figure 3h	One-way ANOVA	Two-tail	F (4, 12) = 4.275
Figure 3i	One-way ANOVA	Two-tail	F (4, 12) = 3.433
Figure 4a	One-way ANOVA	Two-tail	F (4, 11) = 7.425
Figure 4b	One-way ANOVA	Two-tail	F (4, 15) = 25.62
Figure 4c	Student t-test	Two-tail	t=8.058 df=14
Figure 4d	Student t-test	Two-tail	t=3.223 df=8
Figure 4e	Student t-test	Two-tail	t=3.81 df=14
Figure 5b	One-way ANOVA	Two-tail	F (5, 253) = 70.88
Figure 5d	Student t-test	Two-tail	t=8.159 df=57
Figure 5e	Student t-test	Two-tail	t=1.113 df=65
Figure 5g	One-way ANOVA	Two-tail	F (4, 292) = 85.8
Figure 5i	One-way ANOVA	Two-tail	F (4, 10) = 27.1
Figure 5j	One-way ANOVA	Two-tail	F (4, 21) = 8.679
Figure 5k	One-way ANOVA	Two-tail	F (3, 11) = 34.28
Figure 5l	One-way ANOVA	Two-tail	F (3, 8) = 27.69
Figure 5m	One-way ANOVA	Two-tail	F (3, 11) = 64.14
Supplementary Figure 1b	One-way ANOVA	Two-tail	F (4, 10) = 43.56
Supplementary Figure 5a	One-way ANOVA	Two-tail	F (2, 16) = 0.008748
Supplementary Figure 5b	One-way ANOVA	Two-tail	F (2, 15) = 0.2128
Supplementary Figure 6a	One-way ANOVA	Two-tail	F (4, 14) = 106.4

Supplementary Figure 6b	One-way ANOVA	Two-tail	F (4, 38) = 0.7664
Supplementary Figure 8a	Two-way ANOVA	Two-tail	Relax: F (4, 129) = 44.72 Time: F (3, 129) = 113
Supplementary Figure 8b	Two-way ANOVA	Two-tail	Relax: F (4, 166) = 66.65 Time: F (3, 166) = 78.85
Supplementary Figure 8c	Two-way ANOVA	Two-tail	Relax: F (4, 181) = 28.97 Time: F (3, 181) = 41.56
Supplementary Figure 10a	One-way ANOVA	Two-tail	F (4, 20) = 13.29
Supplementary Figure 10b	Student t-test	Two-tail	986s: t=5.778 df=8 412s: t=9.614 df=9 65s: t=2.705 df=9
Supplementary Figure 12a	One-way ANOVA	Two-tail	F (4, 10) = 8.309
Supplementary Figure 12b	One-way ANOVA	Two-tail	F (4, 10) = 7.354
Supplementary Figure 12c	One-way ANOVA	Two-tail	F (4, 10) = 3.185
Supplementary Figure 12d	One-way ANOVA	Two-tail	F (4, 10) = 4.765
Supplementary Figure 12e	One-way ANOVA	Two-tail	F (4, 10) = 7.997
Supplementary Figure 12f	One-way ANOVA	Two-tail	F (4, 10) = 7.116
Supplementary Figure 12g	One-way ANOVA	Two-tail	F (4, 10) = 9.882
Supplementary Figure 12h	One-way ANOVA	Two-tail	F (4, 10) = 2.068
Supplementary Figure 14b	One-way ANOVA	Two-tail	F (5, 173) = 14.66
Supplementary Figure 15a	One-way ANOVA	Two-tail	F (3, 20) = 0.07132
Supplementary Figure 17b	One-way ANOVA	Two-tail	F (2, 116) = 17.64
Supplementary Figure 18	One-way ANOVA	Two-tail	F (3, 11) = 30.63