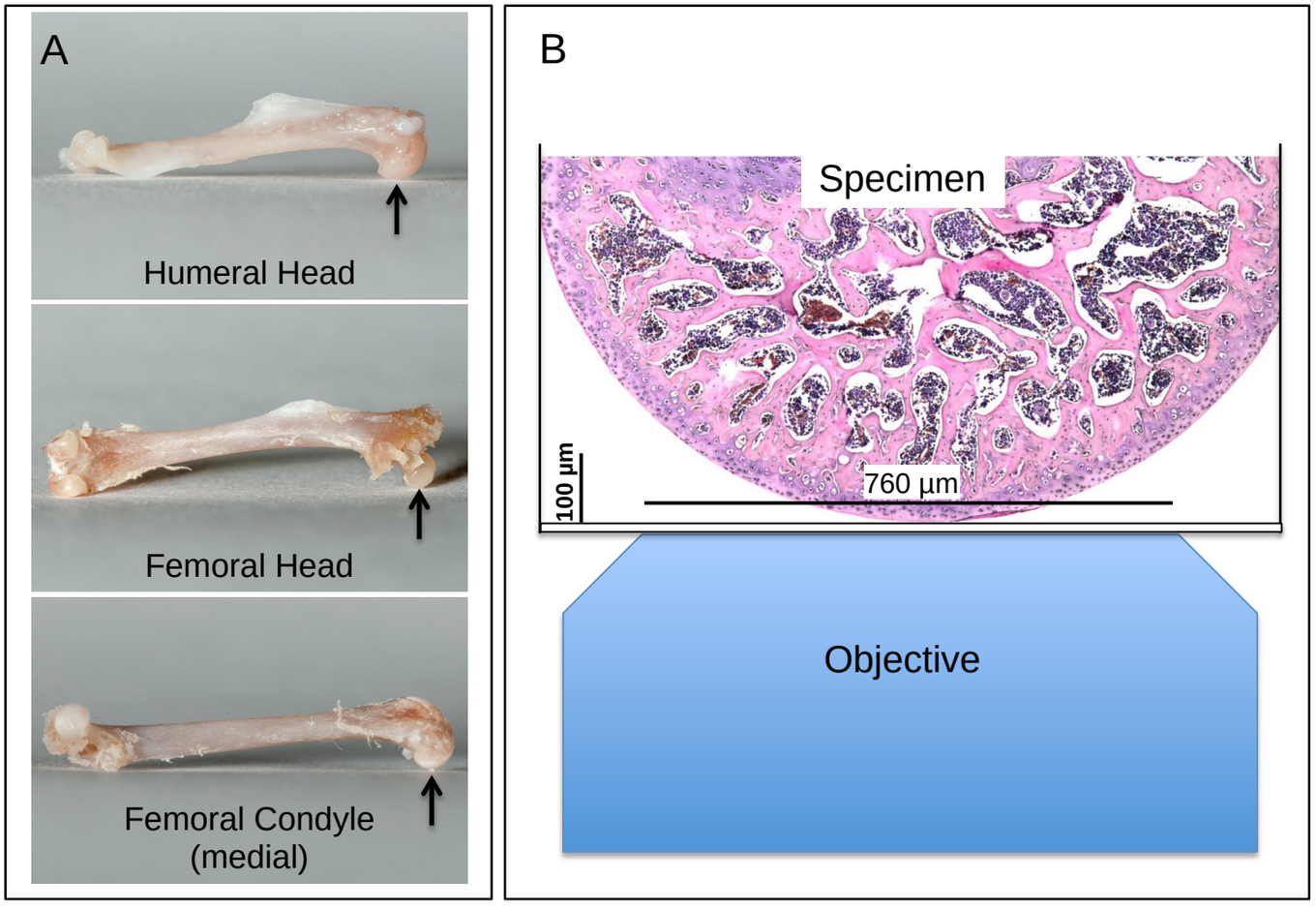
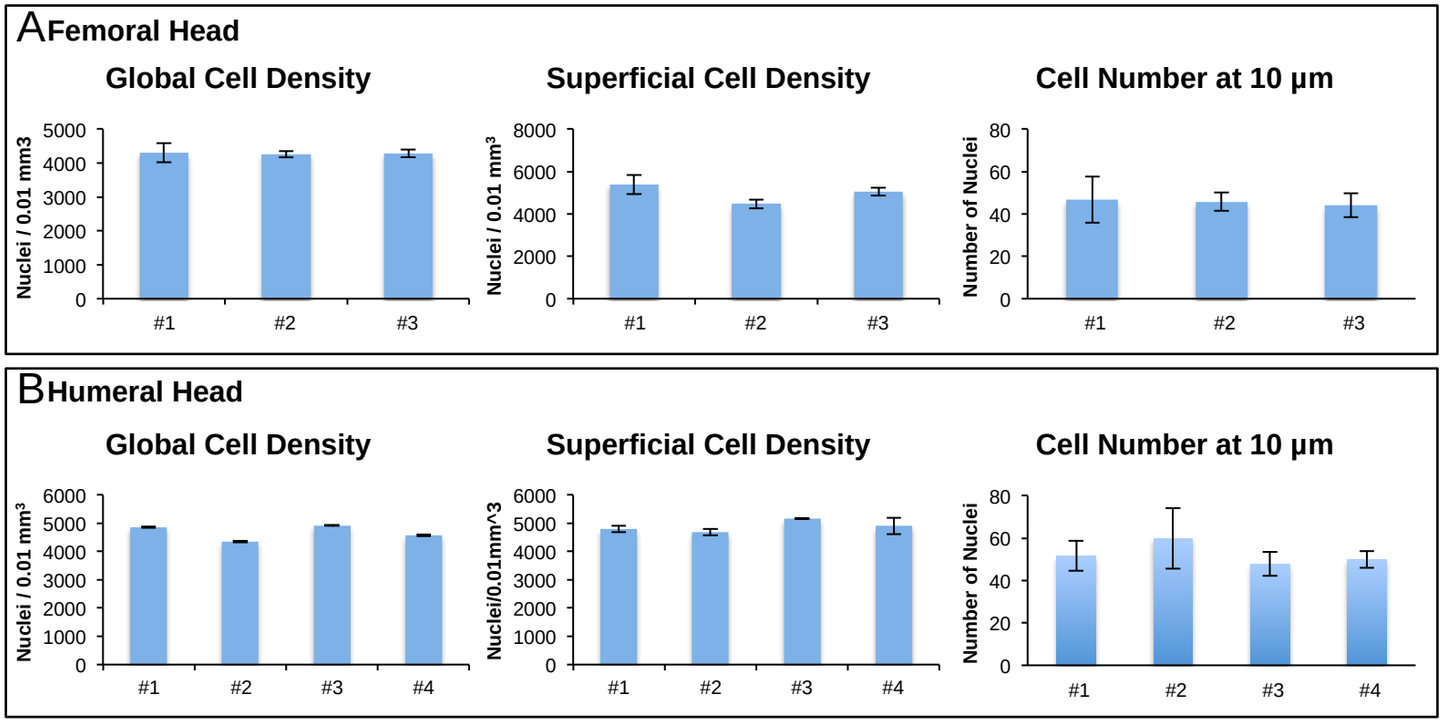


Supplementary Figure 1



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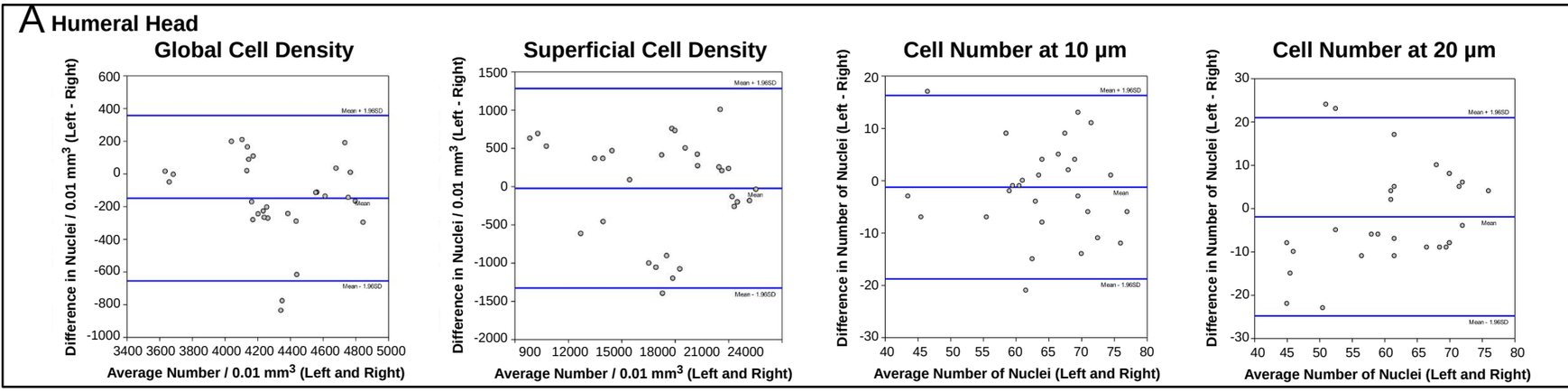
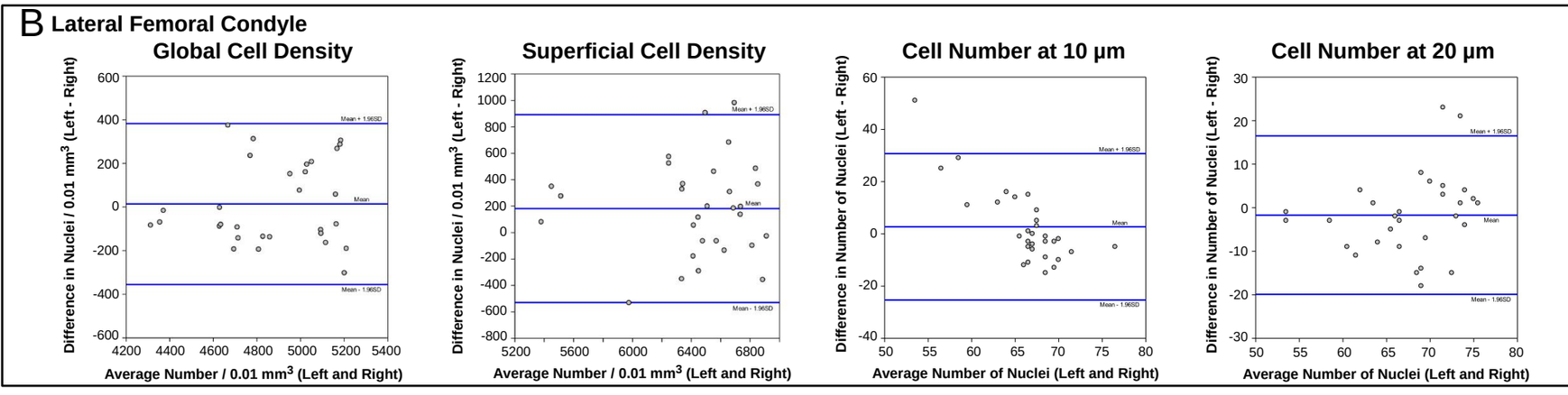
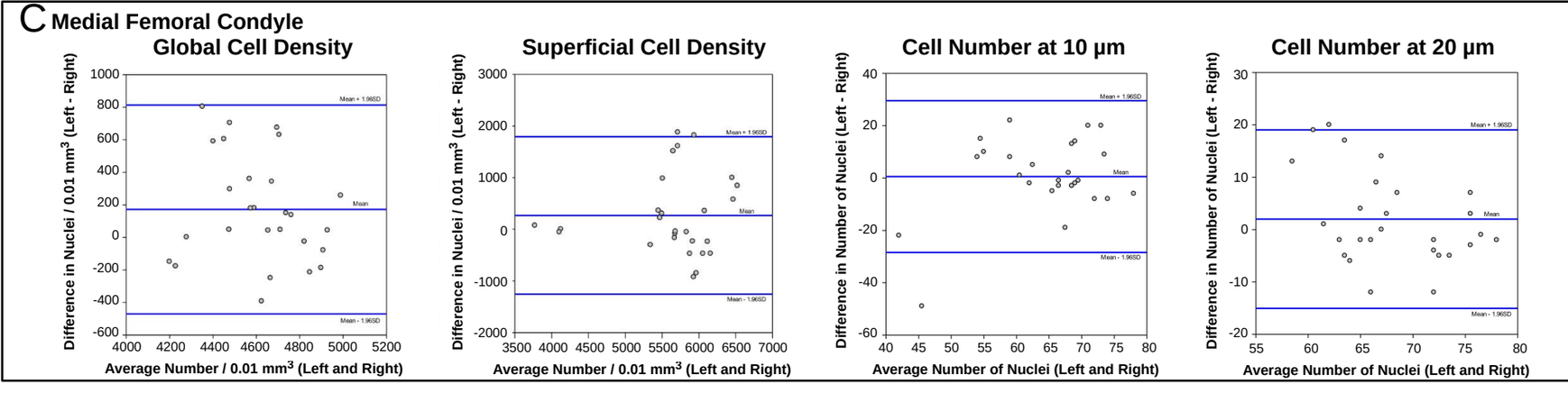
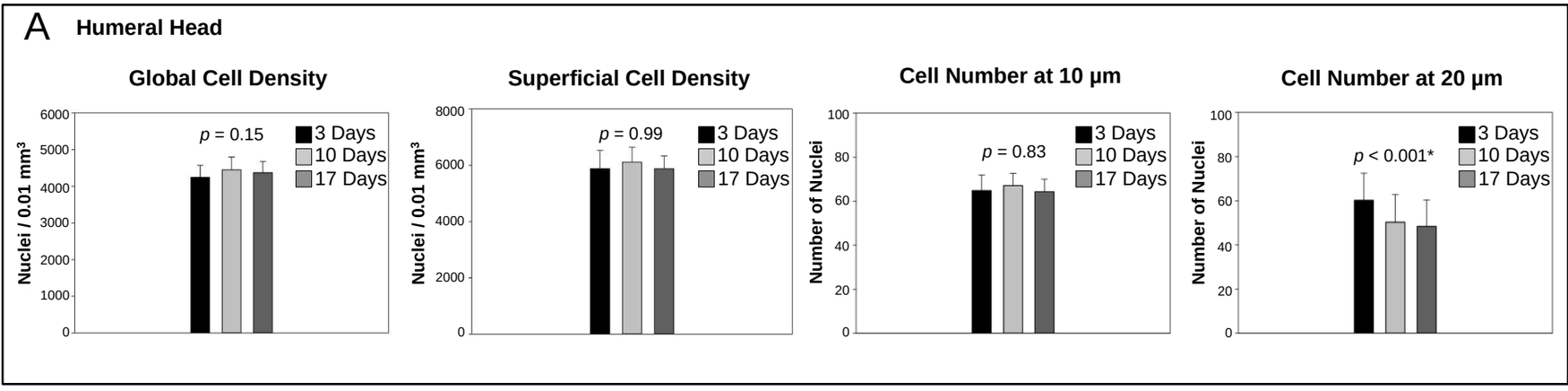
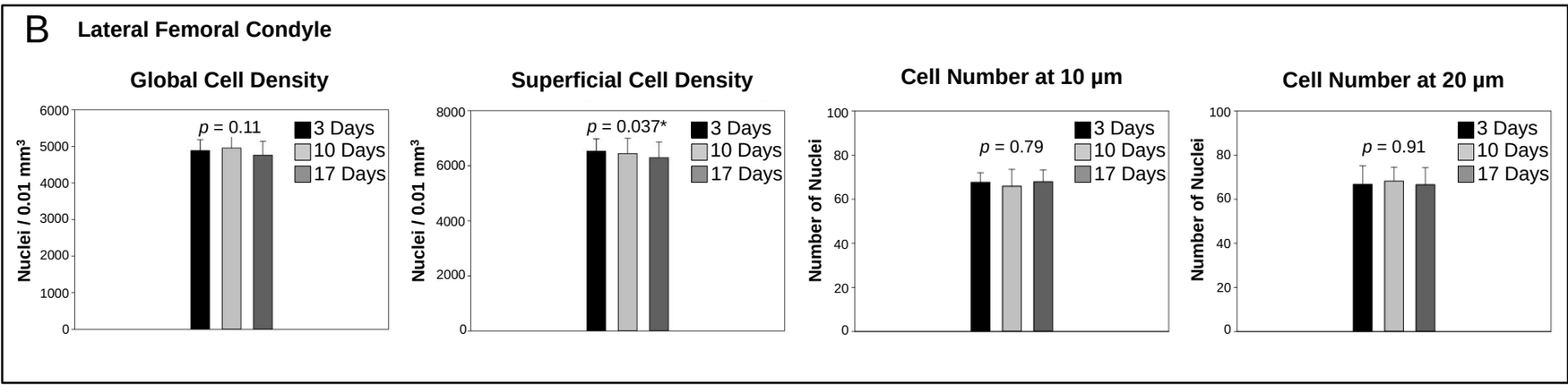


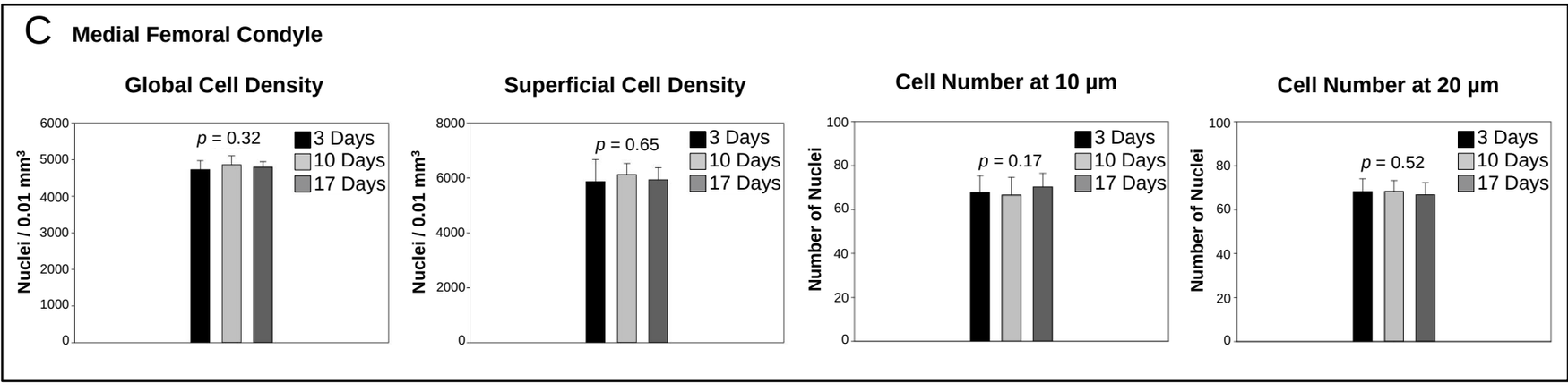




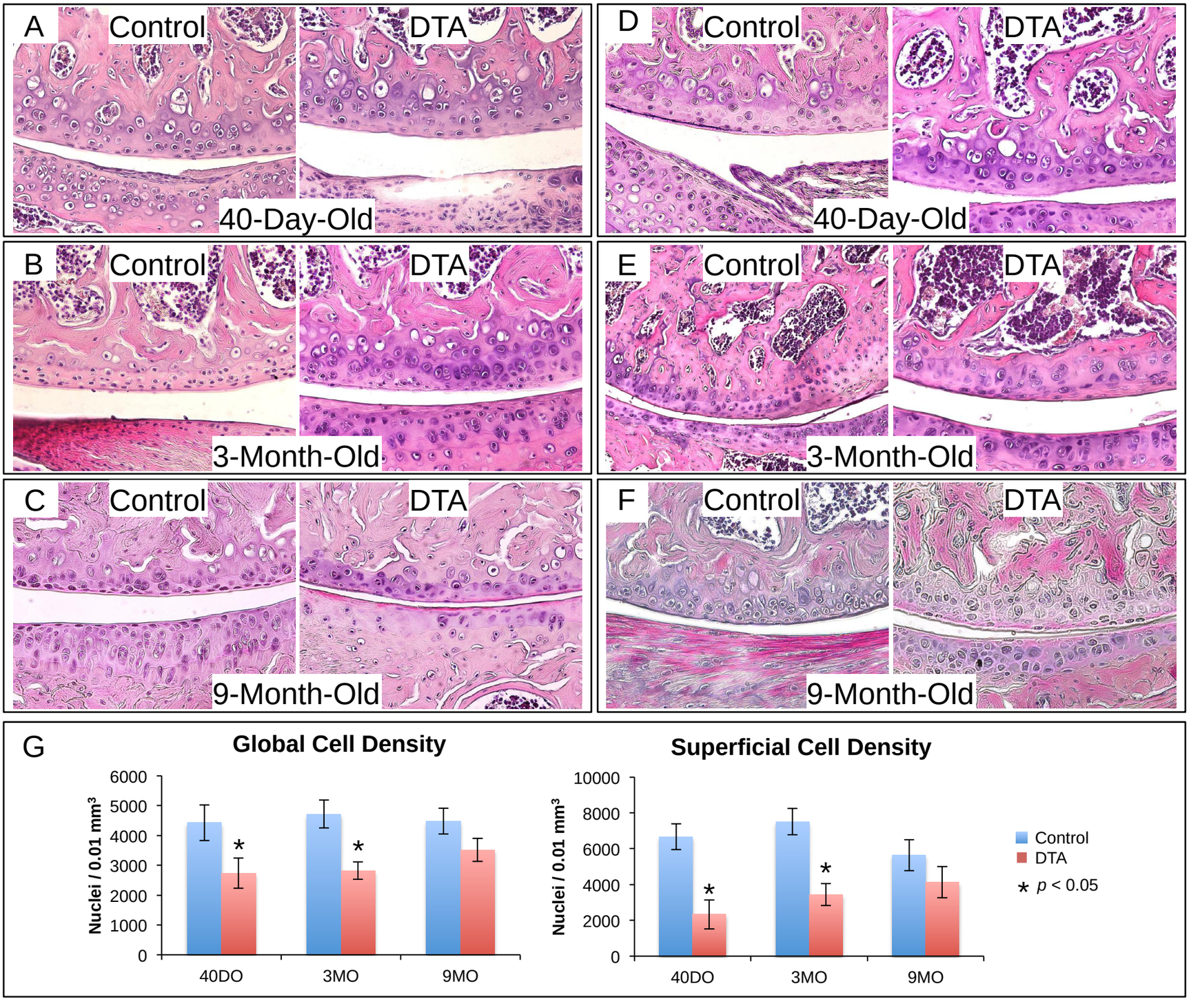
Supplementary Figure 3



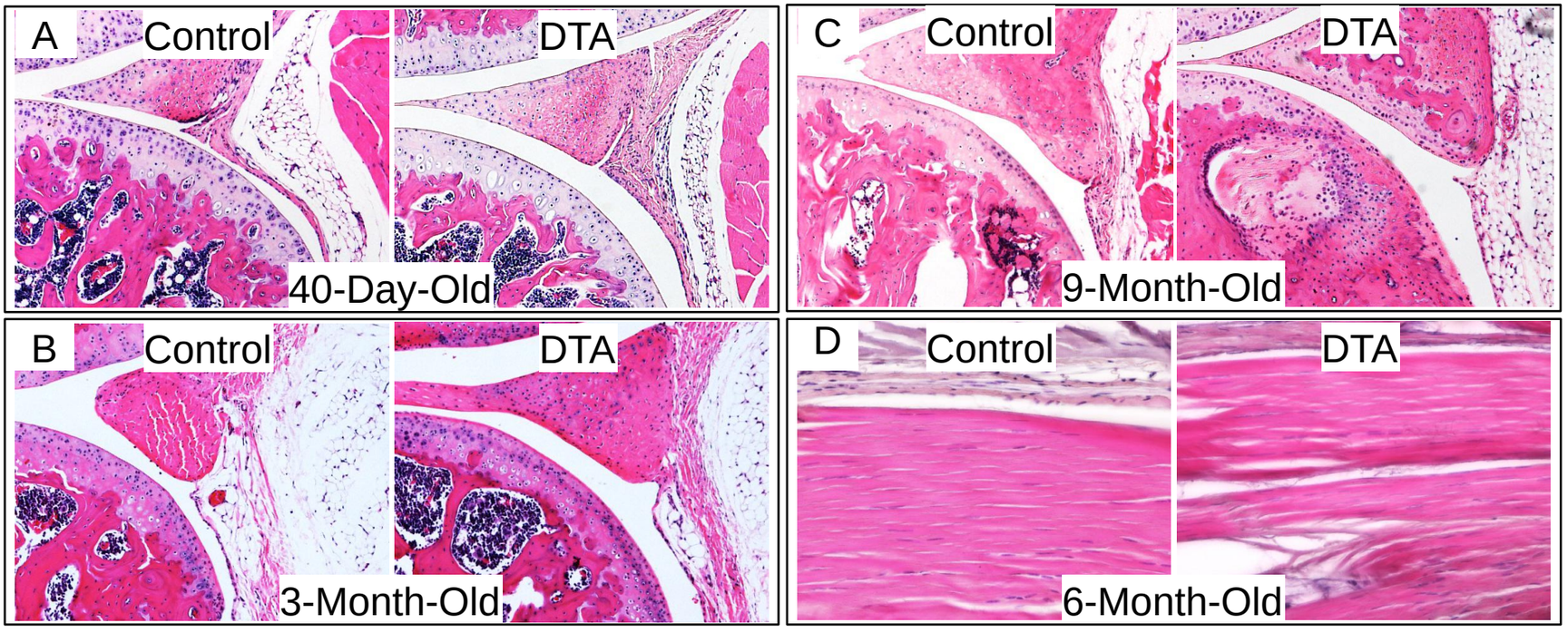




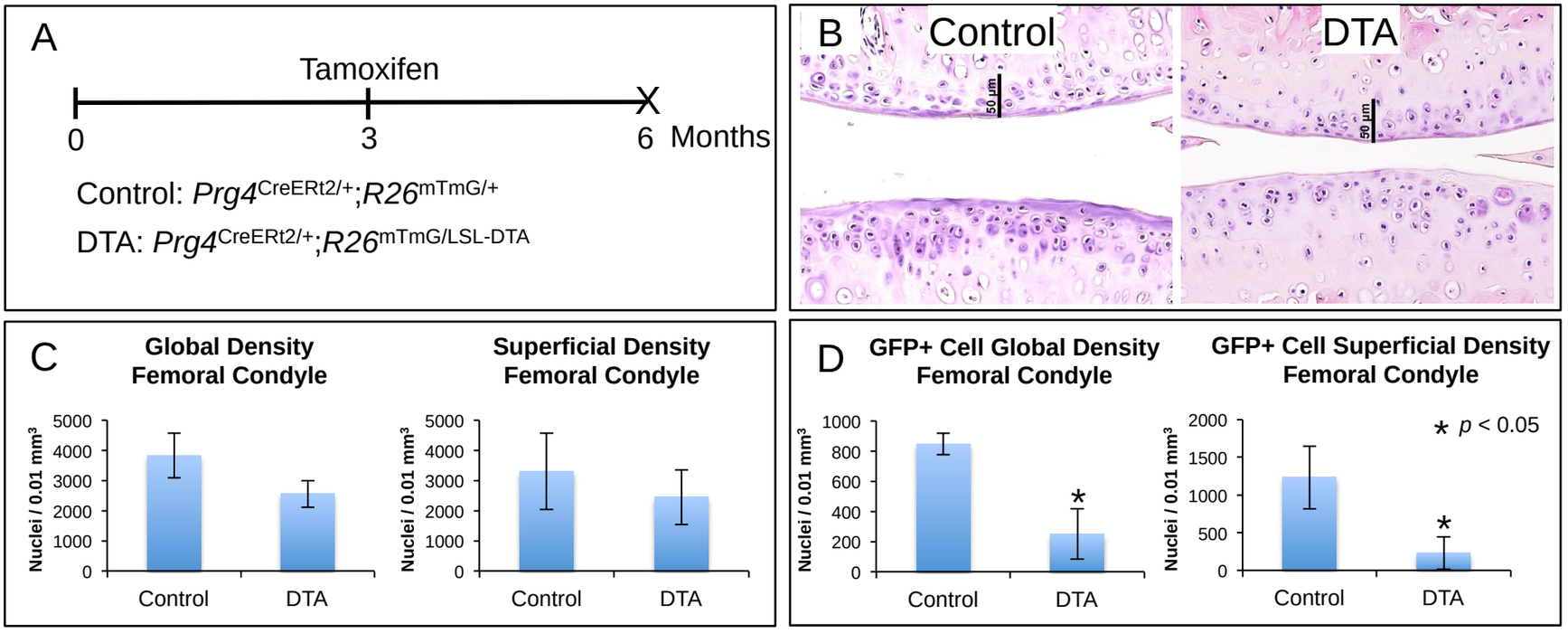
Supplementary Figure 4



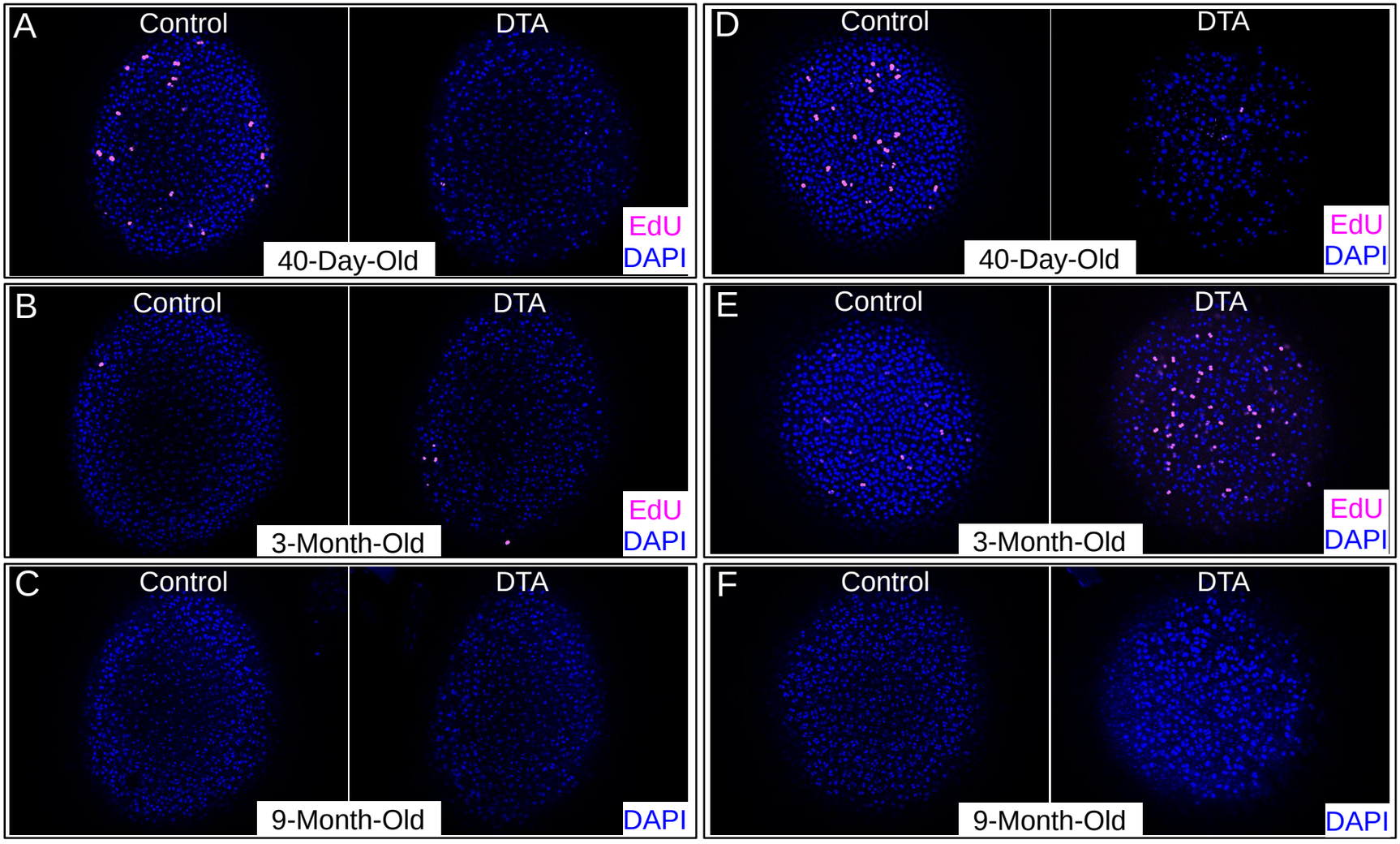
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure 8

Table 1. Bland Altman Analysis of Femoral Head, Humeral Head, Lateral Condyle, and Medial Condyle†

	Bias ± SD	Limits of Agreement	P value
Femoral Head (N = 30)			
Center at 10 um	0 ± 10	-20 to 20	1.00
Center at 20 um	-5 ± 13	-31 to 20	0.036*
Global Density	-20 ± 261	-531 to 491	0.68
Superficial Density	-6 ± 597	-1176 to 1165	0.96
Humeral Head (N = 30)			
Center at 10 um	-1 ± 9	-18 to 16	0.46
Center at 20 um	-2 ± 12	-24 to 21	0.42
Global Density	149 ± 258	-655 to 357	0.004*
Superficial Density	-22 ± 665	-1326 to 1282	0.86
Lateral Condyle (N = 30)			
Center at 10 um	3 ± 14	-25 to 31	0.31
Center at 20 um	-2 ± 9	-20 to 16	0.32
Global Density	14 ± 188	-355 to 383	0.69
Superficial Density	182 ± 363	-529 to 892	0.010*
Medial Condyle (N = 27)			
Center at 10 um	1 ± 15	-28 to 30	0.85
Center at 20 um	2 ± 9	-15 to 19	0.24
Global Density	171 ± 328	-471 to 814	0.012*
Superficial Density	266 ± 778	-1258 to 1790	0.09

SD = Standard Deviation; †P value calculated using a one-sided t-test between left and right side

Supplementary Table 1

Table 2A. Coefficient of Variance for Each Sample of the Femoral Head (N = 30)

Sample ID	Scan 1	Scan 2	Scan 3
Center at 10 μm			
Mean	58	63	60
Standard Deviation	11	7	8
% CV	19.0	11.1	13.3
Center at 20 μm			
Mean	44	41	47
Standard Deviation	15	17	13
% CV	34.1	41.5	28.7
Global Density (0.01 mm³)			
Mean	40×10^2	42×10^2	41×10^2
Standard Deviation	1×10^2	2×10^2	3×10^2
% CV	2.5	4.8	7.3
Superficial Density (0.01 mm³)			
Mean	50×10^2	43×10^2	43×10^2
Standard Deviation	5×10^2	4×10^2	7×10^2
%CV	10.0	9.3	16.3

%CV = Percent Coefficient of Variation

Table 2B. Coefficient of Variance for Each Sample of the Humeral Head (N = 30)

Sample ID	Scan 1	Scan 2	Scan 3
Center at 10 μm			
Mean	63	67	64
Standard Deviation	9	6	6
% CV	14.3	9.0	9.4
Center at 20 μm			
Mean	61	50	48
Standard Deviation	12	13	12
% CV	19.7	26.0	25.0
Global Density (0.01 mm³)			
Mean	42×10^2	45×10^2	44×10^2
Standard Deviation	3×10^2	3×10^2	3×10^2
% CV	7.1	6.7	6.8
Superficial Density (0.01 mm³)			
Mean	59×10^2	61×10^2	59×10^2
Standard Deviation	6×10^2	5×10^2	4×10^2
%CV	10.0	8.2	6.8

%CV = Percent Coefficient of Variation

Table 2C. Coefficient of Variance for Each Sample of the Lateral Condyle (N = 30)

Sample ID	Scan 1	Scan 2	Scan 3
Center at 10 μm			
Mean	68	66	68
Standard Deviation	4	8	5
% CV	5.9	12.1	7.4
Center at 20 μm			
Mean	67	68	67
Standard Deviation	8	6	8
% CV	11.9	8.8	11.9
Global Density (0.01 mm³)			
Mean	49×10^2	50×10^2	48×10^2
Standard Deviation	3×10^2	3×10^2	4×10^2
% CV	6.1	6.0	8.3
Superficial Density (0.01 mm³)			
Mean	65×10^2	64×10^2	63×10^2
Standard Deviation	4×10^2	6×10^2	6×10^2
%CV	6.2	9.4	9.5

%CV = Percent Coefficient of Variation

Table 2D. Coefficient of Variance for Each Sample of the Medial Condyle (N = 30)

Sample ID	Scan 1	Scan 2	Scan 3
Center at 10 μm			
Mean	65	67	70
Standard Deviation	13	8	6
% CV	20.0	14.9	8.6
Center at 20 μm			
Mean	68	68	67
Standard Deviation	6	5	6
% CV	8.8	7.4	9.0
Global Density (0.01 mm³)			
Mean	47×10^2	49×10^2	48×10^2
Standard Deviation	2×10^2	2×10^2	2×10^2
% CV	4.3	4.1	4.2
Superficial Density (0.01 mm³)			
Mean	59×10^2	61×10^2	59×10^2
Standard Deviation	8×10^2	4×10^2	4×10^2
%CV	13.6	6.6	6.8

%CV = Percent Coefficient of Variation

Supplementary Table 2

Table 3A. Sample Size Requirements for Detecting Changes in Femoral Head Measurements†

Variable	Control Mean	Experimental Mean	Difference (Δ)	SD	Percent Change	Effect Size (Δ / SD)	Sample Size	
							80% Power	90% Power
Center at 10µm								
	61	55	6	9	10%	0.7	37	49
	61	49	12	9	20%	1.3	10	13
	61	43	18	9	30%	2.0	6	7
	61	37	24	9	40%	2.7	4	5
	61	31	30	9	50%	3.3	3	4
Center at 20µm								
	44	40	4	15	10%	0.3	222	297
	44	35	9	15	20%	0.6	45	60
	44	31	13	15	30%	0.9	22	29
	44	26	18	15	40%	1.2	12	16
	44	22	22	15	50%	1.5	9	11
Global Density								
	41×10^2	37×10^2	4×10^2	2×10^2	10%	1.7	7	9
	41×10^2	33×10^2	8×10^2	2×10^2	20%	3.4	3	4
	41×10^2	29×10^2	12×10^2	2×10^2	30%	5.1	3	3
	41×10^2	25×10^2	16×10^2	2×10^2	40%	6.8	2	3
	41×10^2	21×10^2	21×10^2	2×10^2	50%	8.4	2	2
Superficial Density								
	45×10^2	41×10^2	5×10^2	6×10^2	10%	0.7	32	42
	45×10^2	36×10^2	9×10^2	6×10^2	20%	1.4	9	12
	45×10^2	32×10^2	14×10^2	6×10^2	30%	2.2	5	6
	45×10^2	27×10^2	18×10^2	6×10^2	40%	2.9	4	4
	45×10^2	23×10^2	23×10^2	6×10^2	50%	3.6	3	4

SD = Standard Deviation; † Based on independent t-test with equal sample sizes

Table 3B. Sample Size Requirements for Detecting Changes in Humeral Head Measurements†

Variable	Control Mean	Experimental Mean	Difference (Δ)	SD	Percent Change	Effect Size (Δ / SD)	Sample Size	
							80% Power	90% Power
Center at 10µm								
	65	59	6	7	10%	0.9	23	30
	65	52	13	7	20%	1.9	6	8
	65	46	19	7	30%	2.7	4	5
	65	39	26	7	40%	3.7	3	3
	65	33	32	7	50%	4.6	3	3
Center at 20µm								
	53	13	5	13	10%	0.4	108	144
	53	13	11	13	20%	0.8	23	31
	53	13	16	13	30%	1.2	12	15
	53	13	21	13	40%	1.6	8	10
	53	13	26	13	50%	2.0	6	7
Global Density								
	44×10^2	39×10^2	4×10^2	3×10^2	10%	1.3	11	14
	44×10^2	35×10^2	9×10^2	3×10^2	20%	2.6	4	5
	44×10^2	30×10^2	13×10^2	3×10^2	30%	3.9	3	3
	44×10^2	26×10^2	17×10^2	3×10^2	40%	5.2	3	3
	44×10^2	22×10^2	22×10^2	3×10^2	50%	6.5	2	3
Superficial Density								
	60×10^2	53×10^2	5×10^2	6×10^2	10%	1.1	15	20
	60×10^2	48×10^2	12×10^2	6×10^2	20%	2.1	5	6
	60×10^2	42×10^2	18×10^2	6×10^2	30%	3.2	3	4
	60×10^2	36×10^2	24×10^2	6×10^2	40%	4.3	3	3
	60×10^2	30×10^2	30×10^2	6×10^2	50%	5.4	3	3

SD = Standard Deviation; † Based on independent t-test with equal sample sizes

Table 3C. Sample Size Requirements for Detecting Changes in Lateral Condyle Measurements†

Variable	Control Mean	Experimental Mean	Difference (Δ)	SD	Percent Change	Effect Size (Δ / SD)	Sample Size	
							80% Power	90% Power
Center at 10µm								
	67	60	7	6	10%	1.1	14	18
	67	54	13	6	20%	2.3	5	6
	67	47	20	6	30%	3.4	3	4
	67	40	27	6	40%	4.5	3	3
	67	34	34	6	50%	5.7	3	3
Center at 20µm								
	67	60	7	7	10%	0.9	21	27
	67	54	13	7	20%	1.8	6	8
	67	47	20	7	30%	2.7	4	5
	67	40	27	7	40%	3.6	3	3
	67	34	34	7	50%	4.5	3	3
Global Density								
	49×10^2	43×10^2	4×10^2	3×10^2	10%	1.4	10	12
	49×10^2	38×10^2	10×10^2	3×10^2	20%	2.8	4	4
	49×10^2	34×10^2	15×10^2	3×10^2	30%	4.2	3	3
	49×10^2	29×10^2	19×10^2	3×10^2	40%	5.6	3	3
	49×10^2	24×10^2	24×10^2	3×10^2	50%	7.0	2	2
Superficial Density								
	64×10^2	58×10^2	6×10^2	5×10^2	10%	1.2	12	16
	64×10^2	51×10^2	13×10^2	5×10^2	20%	2.4	4	5
	64×10^2	45×10^2	19×10^2	5×10^2	30%	3.6	3	3
	64×10^2	39×10^2	26×10^2	5×10^2	40%	4.8	3	3
	64×10^2	32×10^2	32×10^2	5×10^2	50%	6.0	2	3

SD = Standard Deviation; † Based on independent t-test with equal sample sizes

Table 3D. Sample Size Requirements for Detecting Changes in Medial Condyle Measurements†

Variable	Control Mean	Experimental Mean	Difference (Δ)	SD	Percent Change	Effect Size (Δ / SD)	Sample Size	
							80% Power	90% Power
Center at 10µm								
	67	60	7	10	10%	0.7	34	44
	67	54	13	10	20%	1.3	11	14
	67	47	20	10	30%	2.0	6	7
	67	40	27	10	40%	2.7	4	5
	67	34	34	10	50%	3.3	3	4
Center at 20µm								
	68	61	7	5	10%	1.3	12	15
	68	54	14	5	20%	2.5	4	5
	68	47	20	5	30%	3.8	3	3
	68	41	27	5	40%	5.0	3	3
	68	34	34	5	50%	6.3	2	3
Global Density								
	48×10^2	43×10^2	5×10^2	2×10^2	10%	2.2	5	6
	48×10^2	38×10^2	10×10^2	2×10^2	20%	4.3	3	3
	48×10^2	34×10^2	14×10^2	2×10^2	30%	6.5	2	3
	48×10^2	29×10^2	19×10^2	2×10^2	40%	8.7	2	2
	48×10^2	24×10^2	24×10^2	2×10^2	50%	10.8	2	2
Superficial Density								
	60×10^2	54×10^2	6×10^2	6×10^2	10%	1.0	17	22
	60×10^2	48×10^2	12×10^2	6×10^2	20%	2.0	5	7
	60×10^2	42×10^2	18×10^2	6×10^2	30%	3.1	4	4
	60×10^2	36×10^2	24×10^2	6×10^2	40%	4.1	3	3
	60×10^2	30×10^2	30×10^2	6×10^2	50%	5.1	3	3

SD = Standard Deviation; † Based on independent t-test with equal sample sizes

Supplementary Table 3

Supplementary Figure 1. (A) Photographs depicting the orientations of samples when collecting confocal images. Arrows indicate the region of articular cartilage in contact with the glass surface; medial and lateral femoral condyles are imaged separately. **(B)** Diagram illustrating the relative sizes of the microscope aperture and articular cartilage. A histologic section through the humeral head is used to indicate the width and depth of the specimen and the organization of chondrocytes within the specimen. The black bar indicates an imaging plane that is 30 μm below the surface where the cartilage and glass meet. Due to the curvature of the cartilage surface, chondrocytes at the periphery in this plane are nearer to the surface than chondrocytes at the center.

Supplementary Figure 2. Bar graphs depicting the mean (± SD) values for global cell density, superficial cell density, and cell number in the center 100 μm x 100 μm area at 10 μm below the surfaces of the femoral **(A)** and humeral heads **(B)** from individual 1-month-old C57/Bl6J mice (numbered from 1 to 4). Each specimen was oriented and imaged 10 times.

Supplementary Figure 3. Bland-Altman plots comparing measures of cell density (number of chondrocytes/0.01 mm^3 cartilage) across the entire cartilage volume (Global cell density), the superficial volume extending to a depth of 10 μm (Superficial cell density), and cell numbers in the center 100 μm x 100 μm areas at 10 μm and 20 μm depths for the humeral head **(A)**, lateral femoral condyle **(B)**, and medial femoral condyle **(C)**. Each circle represents a comparison between contralateral joint surfaces (n = 10 mice and 3 technical replicates/cartilage). Blue lines indicate the mean ± 1.96 SD, analogous to a 95% confidence interval.

Supplementary Figure 4. Bar graphs depicting mean (± SD) values that were calculated using ANOVA with a generalized estimating equations (GEE) approach for each of the 4 primary measures for the humeral head **(A)**, lateral femoral condyle **(B)**, and medial femoral condyle **(C)** specimens that had been in fixative for 3 days, 10 days, and 14 days (n = 10). When evaluated for directionality of differences between the 3 days, 10 days, and 17 days measurements using a 1-sided t-test, time in fixative was not found to be a significant factor for the superficial femoral density for most measurements. For the few instances when a significant difference was detected between the 3 days and later measurements, (e.g., global cell density of the femoral head), there also was a difference in the coefficient of variance between the measurements performed on different days, indicative of the measurements being influenced by outlying or dissimilar variables. Thus, it was concluded that length of fixation time does not have a significant impact on any of these measurements.

Supplementary Figure 5. (A, B, C) H & E stained sagittal sections (scale bar = 50 μm) through the humeral heads and glenoid fossae of 40-day-old **(A)**, 3-month-old **(B)** and 9-month-old **(C)** control mice (left) and DTA-ablated mice (right). **(D, E, F)** H & E stained sagittal sections (scale bar = 50 μm) through the

femoral heads and acetabuli of 40-day-old **(D)**, 3-month-old **(E)** and 9-month-old **(F)** control mice (left) and DTA-ablated mice (right). **(G)** Bar graphs depicting the mean (± SD) global cell densities and superficial cell densities in the humeral heads of 40-day-old, 3-month-old, and 9-month-old control and DTA-ablated mice (n = 5). Note chondrocyte density measured globally and at the superficial zone decreased significantly (*p < 0.05) in 40-day-old DTA-ablated mice compared to controls

Supplementary Figure 6. (A, B, C) H & E stained knee joint synovium of 40-day-old **(A)**, 3-month-old **(B)** and 9-month-old **(C)** control mice (left) and DTA-ablated mice (right). **(D)** H & E stained tail tendon of 6-month-old control mice (left) and DTA-ablated mice (right).

Supplementary Figure 7. (A) Schematic depicting the experimental timeline for inducing DTA-mediated cell death by administering Tamoxifen for 10 consecutive days at 3 months of age. Animals were analyzed at 6 months of age. **(B)** H & E stained sagittal sections (scale bar = 50 μm) through the tibial-femoral joint of control mice (left) and DTA-ablated mice (right). **(C, D)** Bar graphs depicting the mean (± SD) DAPI stained **(C)** and GFP+ **(D)** global cell densities and superficial cell densities in the lateral femoral condyle of control and DTA-ablated mice (n = 3). Although there were significant decreases in the chondrocyte densities of *Prg4* expressing (i.e., GFP+) cells in the femoral condyles of DTA-ablated mice compared to controls, nuclear cell density did not differ.

Supplementary Figure 8. (A, B, C) Confocal images showing DAPI stained chondrocytes at a depth of 20 μm in the lateral femoral condyles from 40-day-old **(A)**, 3-month-old **(B)**, and 9-month-old **(C)** control mice (left) and DTA-ablated mice (right). **(D, E, F)** Confocal images showing DAPI stained chondrocytes at a depth of 20 μm in humeral heads from 40-day-old **(D)**, 3-month-old **(E)**, and 9-month-old **(F)** control mice (left) and DTA-ablated mice (right). Nuclei of cells that had divided, as indicated by EdU incorporation, are pink in panels **A**, **B**, **D**, and **E**.

Supplementary Table 1. Results of the 1-sided t-tests used to supplement the Bland-Altman analyses on agreement.

Supplementary Table 2. Coefficient of variance values for each measurement obtained after 3 days (Scan 1), 10 days (Scan 2), and 17 days (Scan 3) in fixative for the femoral head, humeral head, lateral femoral condyle, and medial femoral condyle. (n = 10 cartilages and 3 technical replicates per cartilage.)

Supplementary Table 3. Estimations of sample sizes that will be required to detect significant differences between experimental animals and their controls, with respect to cell numbers in the center 100 μm x 100 μm areas at 10 μm and 20 μm depths, global cell density (0.01 mm^3), and superficial cell density (0.01 mm^3) for the femoral head, humeral head, lateral femoral condyle, and medial

femoral condyle. Note that for most measurements, < 10 mice/group are needed to detect a 20% change and < 5 animals/group are needed to detect a 30% change.

Supplemental Methods

3-D Reconstruction of the Articular Cartilage and Quantitative Analysis

1. Load image file into Imaris and calibrate the image pixel size, if necessary.
2. Start a new “Measurement Points” object in “Surpass” view. In “Select” mode, use Shift+Left-click to select excessive soft tissues and ”Set Intensity” to “0” to delete selected objects, if necessary.
3. Surface creation.
   (i) Click on “Add new surfaces” button in the “Object” toolbar.
   (ii) In the wizard opened in the “Object Properties Area”, select the channel that is used to determine the volume. Set “Smooth” at “2 μm”. Select “Background Subtraction” and set it for “7 μm”, which is approximately the diameter of nuclei of the largest object. Click the blue arrow “Next” button to go to next step.
   (iii) Use “Auto Threshold”, deselect “Filters” and finish surface rendering by clicking the green arrow “Finish” button. In the “View Area”, a surface will be overlaid on the 3D image.
   (iv) Click on “Color” and change the color of the new surface to white, in order to better visualize.
   (v) Click on “Edit” (pencil). In the “Select” mode, delete all the out-of-plane objects.
4. Create a channel based on the surface
   (i) In “Edit” panel, select mask all. A window will popup.
   (ii) In this window, select the channel that is used to determine the volume, and set “Voxels Outside Surface” to “0” and “Voxels Inside Surface” to “1”. Click OK and a new channel is created and shown in “Display Adjustment”.
5. Image processing
   (i) Click on “Image Processing" In the main toolbar and select “Channel Arithmetics”.
   (ii) In the popup window, type in “bwconvhull(chX>0)”. Note, X is the number of the channel created in step 4.
   (iii) A new channel is created and can be seen in “Display Adjustment”.
6. Distance transformation
   (i) Create new surface and select the channel created in step 5 as source channel.
   (ii) Uncheck “Smooth” and “Background Subtraction”, and click “Next”.
   (iii) Use “Auto Threshold”, deselect “Filters” and finish creating surface.
   (iv) Use “Clipping Plane” in the “Object” toolbar to visualize the new surface in 3D.
   (v) Click on “Edit” in the main toolbar and select “Change Data Type”. Then select “32 Bit Float” in the popup window.
   (vi) In the wizard in the “Object Properties Area”, click on the “Tool” tab and select “Distance Transformation”.
   (vii) Select “Inside Surface Object” and then the “Distance Channel” is created.

7. Spot detection
   (i) Click on “Add New Spot” button in the “Object” toolbar.
   (ii) Select the channel with DAPI, EdU or GFP labeled cells as source channel. Use “Estimated XY Diameter” at 5 μm for DAPI and EdU and 10 μm for GFP.
   (iii) Use “Quality” filter, set lower threshold at 9 for DAPI and EdU and 1 for GFP, and click on “Finish” button.
   (iv) Click on “Filters” and add “Intensity Center Ch=Y” as a filter. Note, Y is the number of the channel created in step 6.
   (v) The maximum threshold shown in the wizard is the maximum thickness of the object.
   (vi) Set “Lower Threshold” at “0” and “Upper Threshold” at “10” to calculate number of cells in the superficial region; and set “Lower Threshold” at “0” and “Upper Threshold” to maximum to calculate number of cells in the global region”
   (vii) Use “Filters” “Position X”, “Y” or “Z” to calculate number of cells in different regions in the 3D image.
   (viii) Click on “Duplicate Selection to New Spot”, click on “Statistics” then “Detailed”, select the statistics of interest and “Export Statistics”. For example, select “Intensity Center Ch=Y” (Y is the number of the “Distance Channel” created in step 6) to export distance of the center of each nucleus from the surface of the object.
8. Volume Calculation
   (i) Create new surface using the “Distance Channel” as source channel.
   (ii) Uncheck “Smoothing” and “Background Subtraction”.
   (iii) To calculate “Global Volume”, set “0” as “Lower Threshold” and the value of maximum thickness as “Upper Threshold”. To calculate “Superficial Volume”, set “0” as “Lower Threshold” and “10” as “Upper Threshold”.
   (iv) Finish calculation and click on “Statistics”, “Detailed” and “Volume” to see the result.