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9 **Contrast-enhanced microCT evaluation of degeneration following partial and full width injuries to**  
10 **mouse lumbar intervertebral disc**

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22 **Keywords:** Lumbosacral surgical exposure; Intervertebral disc degeneration; annulus fibrosus injury;

23 mouse model

## Partial- and full- width injury of the mouse lumbar IVD

### 24 **Abstract**

25 **Study Design:** Preclinical animal study

26 **Objective:** Evaluation of the degenerative progression resulting from either a partial- or full- width injury  
27 to the mouse lumbar intervertebral disc (IVD) using contrast-enhanced micro-computed tomography and  
28 histological analyses. We utilized a lateral-retroperitoneal surgical approach to access the lumbar IVD, and  
29 the injuries to the IVD were induced by either incising one side of the annulus fibrosus or puncturing both  
30 sides of the annulus fibrosus. The full-width injury caused dramatic reduction in nucleus pulposus  
31 hydration and significant degeneration. A partial-width injury produces localized deterioration around the  
32 annulus fibrosus site that resulted in local tissue remodeling without gross degeneration to the IVD.

33 **Methods:** Female C57BL/6J mice of 3-4 months age were used in this study. They were divided into three  
34 groups to undergo a partial-width, full-width, or sham injuries. The L5/L6 and L6/S1 lumbar IVDs were  
35 surgically exposed using a lateral-retroperitoneal approach. The L6/S1 IVDs were injured using either a  
36 surgical scalpel (partial-width) or a 33G needle (full-width), with the L5/L6 serving as an internal control.  
37 These animals were allowed to recover and then sacrificed at 2-, 4-, or 8- weeks post-surgery. The IVDs  
38 were assessed for degeneration using contrast-enhanced microCT (CE $\mu$ CT) and histological analysis.

39 **Results:** The high-resolution 3D evaluation of the IVD confirmed that the respective injuries localized  
40 within one side of the annulus fibrosus or spanned the full width of the IVD. The full-width injury caused  
41 deteriorations in the nucleus pulposus after 2 weeks that culminated in significant degeneration at 8 weeks,  
42 while the partial width injury caused localized disruptions that remained limited to the annulus fibrosus.

43 **Conclusion:** The use of CE $\mu$ CT revealed distinct IVD degeneration profiles resulting from partial- and  
44 full- width injuries. The partial width injury may serve as a better model for IVD degeneration resulting  
45 from localized annulus fibrosus injuries in humans.

## Partial- and full- width injury of the mouse lumbar IVD

### 46 **Introduction**

47           Mouse models are often used for the preclinical validation of treatments and therapeutic candidates.  
48 Mice offer particular advantages of relatively easy maintenance, year-round breeding with a short gestation  
49 period, delivery of large litters, and inbred tolerance<sup>1</sup>. Furthermore, the ability to manipulate the mouse  
50 genome enables mechanistic studies with greater biological precision than larger mammalian models  
51 including rats, rabbits, and pigs<sup>1</sup>. Moreover, the mouse lumbar intervertebral disc (IVD) provides close  
52 geometric and microstructural semblance to the human lumbar IVD compared to other preclinical animal  
53 models<sup>2</sup>. While the mouse lumbar IVD exhibits age-related degeneration<sup>3,4</sup>, an injury is often utilized to  
54 reproduce the inflammatory conditions and accelerate the IVD's degenerative cascade<sup>5,6</sup>.

55           To create a targeted injury to the IVD, surgical exposure of the IVD is required. These commonly  
56 involve the posterior-lateral, transperitoneal, and anterior/lateral retroperitoneal surgical access. Of these,  
57 the retroperitoneal approach offers multiple advantages: (1) minimal damage to major organs, vessels, and  
58 musculature; (2) access to multiple levels with a relatively small incision; and (3) visual availability of the  
59 target tissue under microscopy. Moss et al. demonstrated the efficacy and reproducibility of the  
60 retroperitoneal approach in rabbits<sup>7</sup>. Exposing the lumbar IVD enables the ability to create a targeted,  
61 directed injury for the investigation of the subsequent degenerative process<sup>8-10</sup>. Masuda et al. and Sobajima  
62 et al. described the rabbit annulus fibrosus injury model where the injury was confined to the annulus  
63 fibrosus that caused slow progressive degeneration of the IVD over 8 weeks. The phenotype and the  
64 progressive nature of this model recapitulates the human disease, and may be better suited to explore  
65 therapies that leverage biological regenerative strategies. In contrast, more damaging approaches that  
66 injures both the annulus fibrosus and nucleus pulposus produce rapid and severe course of degeneration<sup>7,11</sup>.  
67 There are several studies describing IVD degeneration using the lumbosacral IVD injury in the mouse  
68 model<sup>12-14</sup>. In the mouse IVD where the average disc height is approximately 300-400 microns, an injury  
69 by needle puncture produces damage to 55-90% across the height and 15-40% across the width of the  
70 IVD<sup>2,11</sup>, representing significant trauma that is atypical in human IVDs. Moreover, these procedures

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71 involve damage and injury to the nucleus pulposus, such as with a complete puncture to the IVD, with no  
72 possibility to decouple the contributions of the annulus fibrosus and the nucleus pulposus toward the  
73 ensuing degenerative cascade. Piazza and coauthors have shown that unilateral (half-width) and bilateral  
74 (full-width) injuries in the tail IVD results in unique degenerative trajectories, but this has not been  
75 investigated in the mouse lumbar spine. We thus sought to compare the degenerative profiles of IVDs after  
76 partial- and full- width injuries in the mouse lumbar spine. In order to evaluate the degeneration of the IVD  
77 in a spatially robust manner, we utilized contrast-enhanced microCT<sup>15,16</sup>, in addition to histological grading,  
78 to quantify the changes in structure and composition at 2-, 4- and 8- weeks after surgery.

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### 79 **Materials and Methods**

#### 80 *Animal Preparation*

81 All procedures were performed following Washington University School of Medicine IACUC  
82 approval. Female C57BL/6J mice of 3-4 months age were used (BW: 20 – 25 g). They were housed under  
83 standard animal husbandry conditions (in a temperature-controlled [ $21 \pm 1^\circ\text{C}$ ] room with normal 12-hr  
84 light/dark cycles). These animals were divided into three groups: Partial-width injury (PW), full-width  
85 injury (FW), and Sham (n=15-18 per group) with all animals undergoing to retroperitoneal surgical  
86 exposure of the lumbar IVD. The groups were cross-sectionally evaluated at 2-, 4-, and 8- week post-  
87 surgery time points. The injury was delivered to the L6/S1 IVD with the L5/6 IVD used as the internal  
88 control.

89 The partial-width injury mimics a localized injury to the annulus fibrosus, aka annular tear injury, in  
90 humans<sup>17</sup>. To evaluate the feasibility of this injury, we first performed the partial-width injury on six  
91 animals, and then they were euthanized shortly after recovery from anesthesia. The IVDs were harvested  
92 and measured for the thickness of the annulus fibrosus and the depth of the injury using contrast-enhanced  
93 microCT (CE $\mu$ CT) and histological analysis.

94 The second set of animals were allowed to recover following PW, FW or Sham surgery for 2, 4 or 8  
95 weeks (n = 5-6 per group) and then euthanized, and the IVD tissues were assessed for degeneration with  
96 CE $\mu$ CT and histological analysis. Samples where attenuation was saturated were not included in analysis.

97 Mice were anesthetized with isoflurane gas in oxygen via a facemask (3–4% induction and 2–2.5%  
98 maintenance at 1 L/min flow rate; Highland Medical Equipment) and were given a preoperative intradermal  
99 injection of lidocaine (7 mg/kg; Hospira, Inc). The left flank was then shaved from the ventral to the dorsal  
100 midlines, and the skin was sterilized. The skin was prepared for aseptic surgery via washing with 70%  
101 ethanol and povidone iodine.

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102 *Partial-width injury:* A distance of 0.3 mm from the end of the No. 11-scalpel blade tip was measured  
103 and marked with a micro-caliper under a microscope (Figure 1). The distance of 0.3 mm was determined  
104 from our preliminary studies. The edge of the blade was allowed to insert into the IVD until the 0.3 mm  
105 marking was no long visible under the microscope. Once pierced, the injury site was closely observed  
106 under the microscope to confirm that there is no leakage of the NP.

107 *Full-width injury:* A 33G needle was inserted bilaterally through the IVD lateral axis of the IVD. In  
108 contrast to the partial-width injury, NP herniations were observed following the full-width injury.

109 *Contrast-enhanced microCT tomography (CE $\mu$ CT):* Samples were incubated in a solution of 175  
110 mg/mL solution diluted from a stock of OptiRay 350 (Guerbet, St. Louis) in PBS at 37°C. After 24 hrs of  
111 incubation, samples were scanned using a  $\mu$ CT40 (Scanco Medical, CH) at a 10- $\mu$ m voxel size (45 kVp,  
112 177 uA, high resolution, 300 ms integration).

113 CE $\mu$ CT data was exported as a DICOM file for analysis in a custom MATLAB program. After an  
114 initial median filter (sigma = 0.8, support = 5), functional spine units were isolated from surrounding soft  
115 tissue not part of the IVD by drawing a contour around the outer edge every 5 transverse slices and  
116 morphing using linear interpolation. The IVD was manually segmented from the vertebral bodies with the  
117 same methodology as above. The remaining voxels were designated as the whole disc mask. The NP was  
118 thresholded from the AF followed by a morphological close and morphological open to fill interior holes  
119 and smooth the boundary. The volumes and average attenuations (intensity) were calculated from the mask  
120 of the NP and whole disc. The volume was determined from the total number of voxels contained within  
121 the mask and the attenuation was taken as the average 16-bit grayscale value of the voxels. Visualizations  
122 of the microCT were obtained using the image processing application OsiriX (Pixmeo, Geneva). AF  
123 thickness, partial-width injury depth and disc height index (DHI) were measured along the mid-sagittal  
124 plane. DHI was calculated as the ratio of the IVD height to width. IVD height was taken as the average at  
125 5 equidistant points along the mid-sagittal plane. The ratio of NP intensity/disc intensity (NI/DI), defined  
126 as the average attenuation of voxels in the NP mask divided by the full disc mask, is an unbiased, fully

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127 three-dimensional measure that quantifies the relative size and hydration to inform the relative changes in  
128 degeneration<sup>18</sup>.

129 *Histological analysis:* Following microCT, samples were fixed for 24 hours in 10% neutral buffered  
130 formalin followed by 3 days of decalcification in Immunocal (StatLab 1414-X). The samples were  
131 embedded in paraffin, sectioned at a thickness of 10  $\mu\text{m}$ , and then stained with Safranin-O and Fast Green.

132 *Measuring thickness of AF and the depth of partial-width injury:* The CE $\mu$ CT of the injured L6/S1  
133 and histological analysis on uninjured L5/6 were used to measure the thickness of the AF. The CE $\mu$ CT on  
134 the injured L6/S1 was used to measure the depth of injury as defined by the shortest perpendicular distance  
135 from the outer edge of annulus fibrosus to the visually observable outline of the injury site.

136 *Assessment of IVD degeneration:* Histological classification system recently developed by Melgoza  
137 et al. in 2021 was used to quantify the degeneration of the injured level (L6/S1) which allows the  
138 independent evaluation of the nucleus pulposus, annulus fibrosus, endplates and interface boundaries<sup>19</sup>.  
139 Morphology and NI/DI determined from CE $\mu$ CT was used to further inform the level of degeneration of  
140 the injured level (L6/S1) compared to the internal control (L5/6).

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### 141 **Results**

142 A total of 57 mice were subjected to the surgical procedure. The average surgery time was 15 min 38 sec ±  
143 6 min 23 sec from incision to closure. There was no mortality or severe complication such as massive  
144 bleeding due to vascular injury during surgery. Caution was taken to prevent injury of the lumbosacral  
145 plexus located posteriorly during the blunt dissection of the psoas muscle from posterior to the anterior  
146 direction.

### 147 *Annulus Fibrosus (AF) Thickness*

148 CE $\mu$ CT and histology measurements of AF thickness and IVD width were highly consistent. The  
149 anteroposterior IVD width measured with CE $\mu$ CT was  $1.27 \pm 0.13$  mm (mean  $\pm$  standard deviation),  
150 whereas that measured by histological analysis was  $1.21 \pm 0.11$  mm. AF thickness measured with CE $\mu$ CT  
151 was  $0.38 \pm 0.05$  mm and that measured with histological analysis was  $0.43 \pm 0.10$  mm (Figure 3). The  
152 CE $\mu$ CT measured values were statistically indistinguishable from ( $p = 0.37$ ) and were highly correlated ( $r^2$   
153 = 0.96) with the histologically measured values (Figure 2).

### 154 *Depth of Partial-width Injury*

155 All injuries except one were isolated to the AF (Figure 3B). In sample 5, the NP may have been injured  
156 because CE $\mu$ CT showed disruption of the AF/NP boundary (Figure 3C). The depth of injury measured with  
157 CE $\mu$ CT was  $0.29 \pm 0.05$  mm. The ratio of the injury depth to the AF thickness was  $0.80 \pm 0.19$  (Figure 3D  
158 and 3E).

### 159 *Structural evaluation of the IVD*

160 Neither the partial-width nor the full-width injuries resulted in changes in the Disc Height Index  
161 across the two-, four-, and eight- week time points (Figure 4D). Similarly, the size of the nucleus pulposus,  
162 characterized by the nucleus pulposus (NP) volume fraction, were not dramatically different between  
163 groups and time points (Figure 4E). The relative hydration of the NP, quantified by the ratio of NP  
164 attenuating intensity to that of the whole disc (NI/DI), revealed the full-width injury caused a dramatic



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165 decrease in the attenuation of the NP at the two- and four- week time points, with a trending change at  
166 eight- weeks. Reduced NI/DI indicates a loss of NP hydration.

167 The impact of the partial- and full- width injuries to the annulus fibrosus was observable at two  
168 weeks, The relative hydration of the annulus fibrosus (AF) increases after an injury, and this is evident in  
169 both the partial- and full- width groups. Two weeks after injury, both the partial- and full- injuries showed  
170 an increase attenuation of the AF, with the full-width injury group sustaining this increase (Figure 4G).

171

### 172 *Extent of IVD Degeneration*

173 Quantitative histological analyses revealed a differential degenerative cascade to the IVD following  
174 full-width injury as early as two weeks post injury but not with the partial-width injury. The histological  
175 classification showed significant degeneration following FW injury at all timepoints ( $p < 0.05$ ) in the NP,  
176 AF, interface boundaries, and total IVD score but not endplate score compared to Sham while no differences  
177 were detected between PW and Sham (Figure 5). No morphological differences between timepoints or  
178 injury groups were observed.

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### 179 **Discussion**

180 Animal models of IVD degeneration are imperative to elucidate the key molecular mechanisms of  
181 the pathophysiology. To date, rodents, rabbits, dogs, goats, sheep, and primates have been used as models  
182 for intervertebral disc degeneration<sup>20</sup>. One main advantage of mouse models is the availability of reagents  
183 and modifications that could be used concomitantly with surgically induced IVD degeneration. However,  
184 the small size of the mouse requires a high degree of surgical precision, particularly with access and  
185 exposure of the lumbar spine. While many studies utilize the mouse tail for degenerative models<sup>12,21-23</sup>,  
186 the lumbar spine maintains the anatomical proximity to physiologically relevant structures such as the  
187 dorsal root ganglions and may better recapitulate the human disease<sup>22</sup>.

188 We describe here a novel procedure for mouse lumbosacral IVD injury with visual guidance via  
189 microscopy and gross features. The retroperitoneal space of the spine can be located (Figure S1) with the  
190 left pelvic bone with gluteus muscles and proximal thigh as landmarks (Figure 1C and 1D). Blunt-dissection  
191 of the fat pad surrounding the thoracic cage anteriorly, the gluteus muscles superiorly, and the thigh muscles  
192 posteriorly (Figure 1B) exposes these two landmarks (Figure 1C). The lateral approach to the IVD allows  
193 for precise injury to the annulus fibrosus of the IVD (Figure 1). Exposure of the pelvic bone from the gluteus  
194 muscle and rotation of the pelvic bone posteriorly is necessary to achieve ample exposure of multiple IVDs  
195 in the surgical field. The psoas muscles can be easily scraped posterior-anteriorly with a Penfield dissector.  
196 CE $\mu$ CT allowed for a 3D spatially unbiased quantification of morphology and composition<sup>18</sup> that confirmed  
197 the localization of injury across the cross-section time points. CE $\mu$ CT measurements of the IVD  
198 morphology were in excellent agreement with histological measures. NI/DI and histological analysis  
199 indicated degeneration of injured IVDs starting 4 weeks post-surgery.

200 The present study describes a surgical procedure to expose the lumbosacral spine of mice and to  
201 induce a localized experimental injury with AF-limited depth and compared the degenerative response with  
202 a more commonly used needle puncture injury. The technique described herein also focused on achieving  
203 a consistent depth and size of injury. The marked tip of the No. 11-scalpel blade tip was confirmed with

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204 CE $\mu$ CT to provide AF-limited injury and resulted in no NP leakage under observation by microscopy. The  
205 various sizes of the needle may produce inconsistent depth and size of injury. A thick needle may lead to  
206 end-plate injury due to the small IVD height in mice, whereas the excessive flexibility of a thin needle  
207 would contribute to inconsistency in the depth of injury. While most injury models cause damage to the  
208 nucleus pulposus, the cartilaginous endplates, and even the vertebral body<sup>24-26</sup>, the partial-width injury here  
209 results in an isolated injury to the outer annulus fibrosus. Surprisingly, we observed no degenerative  
210 changes in the endplate in the full-width injury, suggesting that it is possible to induce an IVD only injury  
211 using a carefully applied 33G needle. Following full-width injury, IVDs exhibited quick and sustained  
212 degeneration as early as two weeks after injury, and this is sustained through the four- and eight- week time  
213 points. Despite maintaining disc height, the FW injury caused consistent degenerative changes in the  
214 nucleus pulposus, annulus fibrosus, and interface boundaries which included the AF-endplate and NP-AF  
215 boundaries. The PW injury did not produce statistically significant degenerative changes, but whether the  
216 innervation or vascularization profile changes particularly at the outer annulus fibrosus remains to be  
217 investigated.

218 CE $\mu$ CT allowed for visualization of the injury site following both partial- and full- width injuries.  
219 The increased attenuation on CE $\mu$ CT may indicate changes in composition or diffusion properties resulting  
220 from the disruption in the annulus fibrosus with both injuries. The injured annulus fibrosus appears to be  
221 highly hydrated, due to the increased interstitial fluids that localize around the injury site (Figures 3 -4).  
222 Since IVD degeneration in humans often starts as a tear in the annulus fibrosus<sup>17</sup>, this model will enable  
223 mechanistic investigations of how injuries of the annulus fibrosus contribute to IVD degeneration and the  
224 subsequent development of low back pain. In contrast, the full-width injury to the nucleus pulposus induces  
225 a more rapid and severe course of degeneration that is more aligned with an acute trauma<sup>7,20,21</sup>. Unlike the  
226 increased attenuation of the injured annulus fibrosus, the depressurized nucleus pulposus loses water and  
227 attenuates less than the healthy state (Figures 4). Consistent with unilateral injuries of the tail IVD, the  
228 resultant degenerative cascade was nuanced<sup>11</sup>, and required high resolutions modalities to detect measurable

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229 changes. Future studies will evaluate the partial-width injury as a slowly progressing IVD degeneration  
230 model following a clinically relevant injury.

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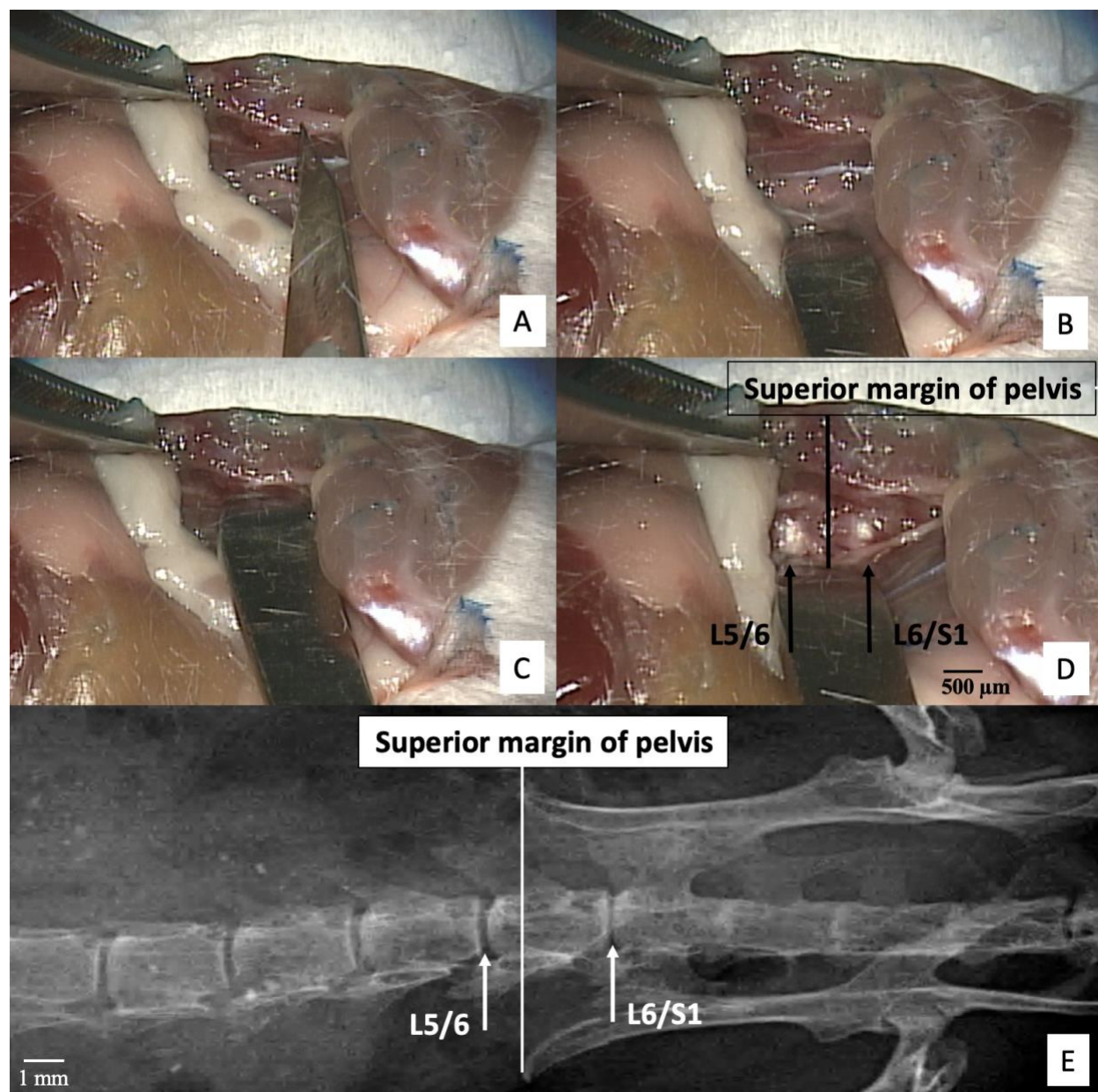
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- 306

307 **Figures**



308  
309 **Figure 1.** *Retroperitoneal dissection to expose the lumbosacral intervertebral disc using microscopic*  
310 *guidance*

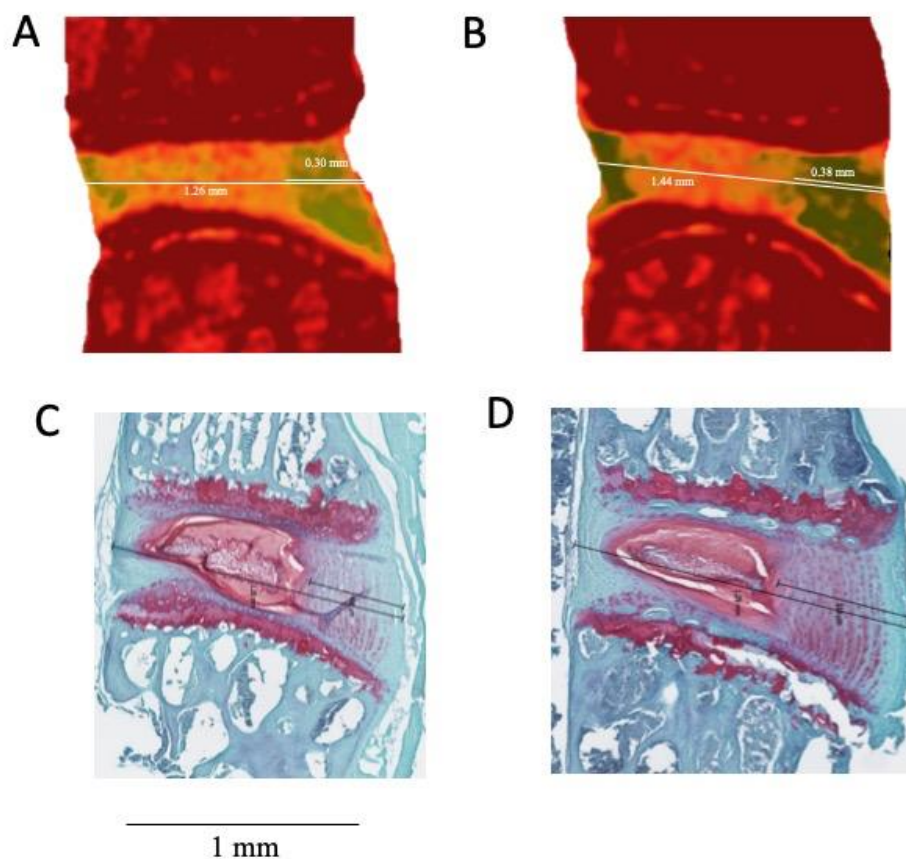
311 (A) The No. 11-scalpel blade indicates the left pelvic bone and forceps grip the gluteus muscles. (B) The  
312 pelvis can be rotated posteriorly to increase working space by raising the gluteus muscles and rotating the  
313 left pelvic bone. The abdominal wall and peritoneum are retracted anteriorly by a Penfield dissector to

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314 expose the psoas muscles. **(C)** The psoas muscle can be stripped posteriorly-to-anteriorly by scraping out  
315 muscles attached to the anterior surface of the pelvis with a Penfield dissector. **(D)** Superior margin of the  
316 pelvic bone indicating the L6 vertebral body. **(E)** X-ray confirms the position of the pelvis relative to the  
317 L5/6 and L6/S1 intervertebral discs.



## Partial- and full- width injury of the mouse lumbar IVD

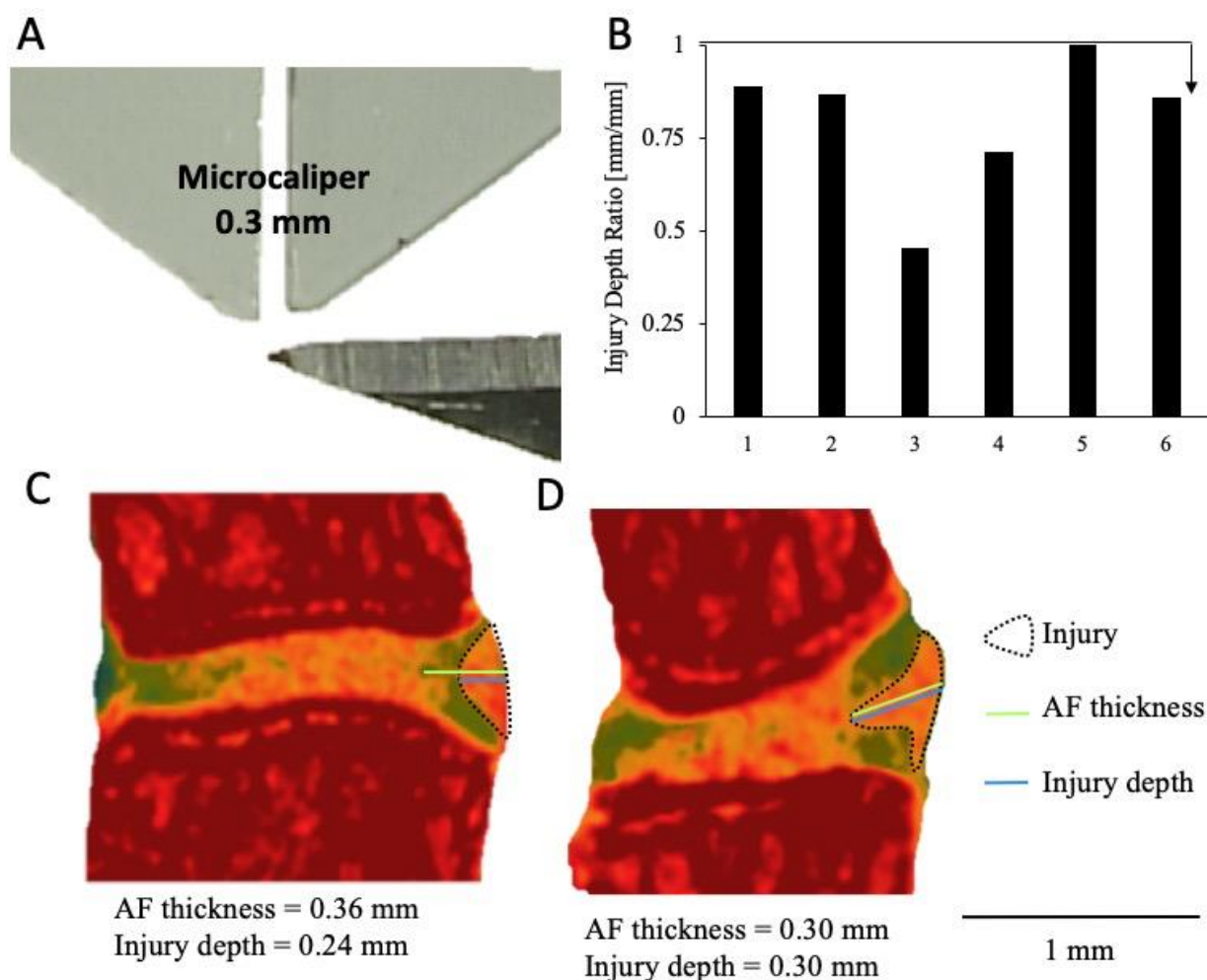


318

319 **Figure 2.** *Measuring the thickness of total intervertebral disc and the annulus fibrosus*

320 **(A-B)** Contrast-enhanced microCT (CEμCT) and **(C-D)** histology were used to measure the IVD widths  
321 and anterior AF thicknesses. The CEμCT and histology measurements were highly correlated ( $r^2 = 0.96$ ,  $p$   
322  $< 0.001$ ) and statistically indistinguishable (paired t-test,  $p = 0.37$ ), confirming the fidelity of the non-  
323 destructive evaluation of CEμCT.

### Partial- and full- width injury of the mouse lumbar IVD



324

325 **Figure 3.** Depth of the partial-width injury measured by contrast-enhanced micro-computed tomography

326 (A) The tip of the scalpel edge is marked at 0.3 mm to allow for visual confirmation during surgery that the

327 partial-width injury is limited to the AF. (B) All of the injury depths were confirmed by CE $\mu$ CT and

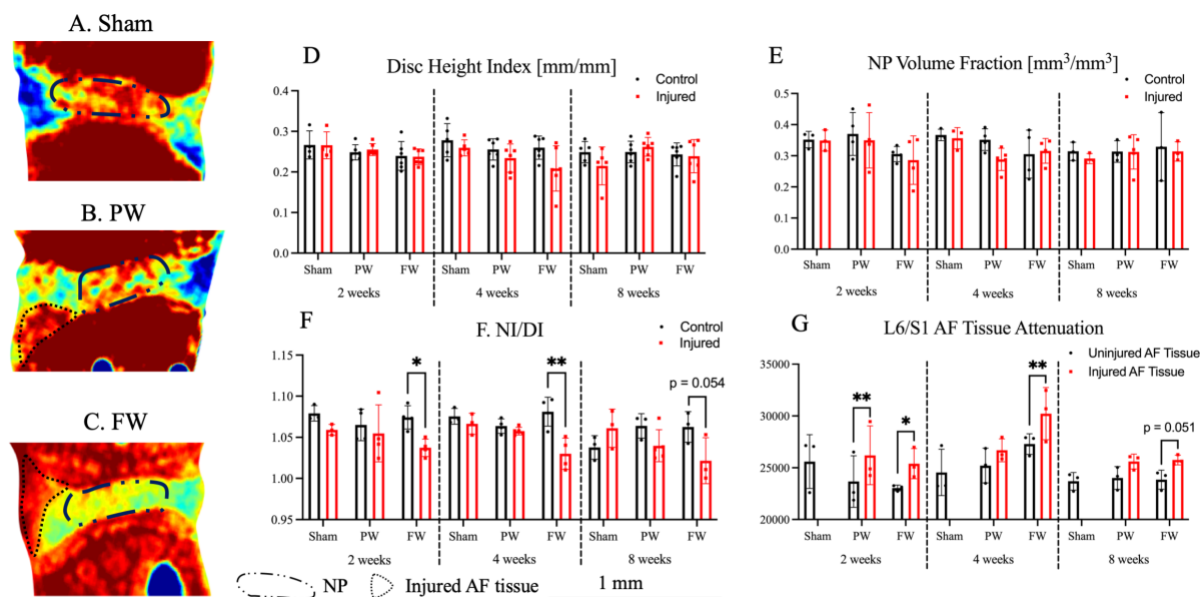
328 histology to have depths that do not exceed of the AF in each injured IVD, confirmed by the ratio of injury

329 depth to AF thickness which are less than 1 in all samples. (C, D) Representative CE $\mu$ CT showing the

330 range of depths achieved by this AF injury.

Partial- and full- width injury of the mouse lumbar IVD

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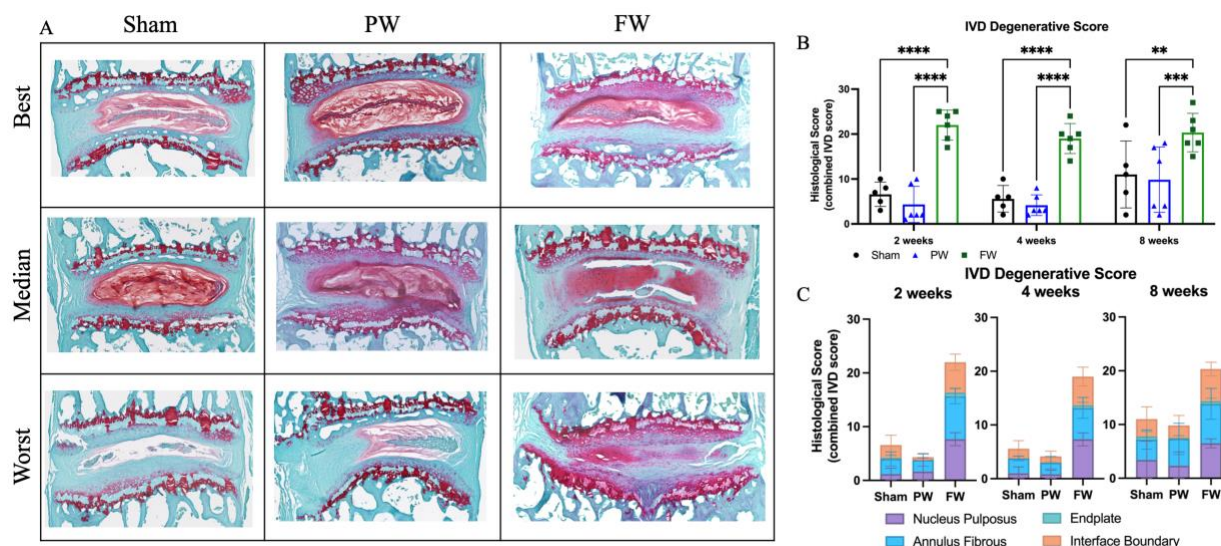


332

333 **Figure 4.** Full-width (FW) injury induces a sustained loss of NP hydration compared to both the partial-  
 334 width (PW) injury and Sham groups.

335 (A-C) Contrast-enhanced microCT of injured level (L6/S1) where injury site is visible in both FW and PW  
 336 groups. (D) Mean attenuation of the AF in the injured and uninjured groups. Paired t-test indicates  
 337 significant increase in attenuation at injury site compared to rest of uninjured AF ( $p < 10^{-5}$ ). (E) NI/DI of  
 338 injured level (L6/S1) and uninjured control level (L5/6). While no changes in the whole IVD structure were  
 339 detected between injured and uninjured levels with either FW or PW, the ratio of the nucleus pulposus  
 340 intensity to the whole disc intensity (NI/DI – approximately 4% drop in intensity) revealed the loss  
 341 hydration and proteoglycans in the nucleus pulposus of FW samples that is indicative of degeneration  
 342 compared to the control IVDs.

Partial- and full- width injury of the mouse lumbar IVD



343  
 344 **Figure 5.** FW induces quick and sustained IVD degeneration compared to both Sham and PW.  
 345 (A) Safranin-O stained histological section of injured level (L6/S1) (B) Total IVD degenerative score of  
 346 L6/S1. (C) Breakdown of NP, AF, endplate and interface boundary contributions to total IVD degenerative  
 347 score. We were unable to visualize the injury on the histological sections after the 2-, 4- and 8- week  
 348 recovery timepoints. Significant degeneration was observed at all timepoints indicated by significant  
 349 increases in NP, AF, interface boundary and total IVD degenerative scores in the FW group compared to  
 350 Sham and PW ( $p < 0.05$ ). While PW causes localized disruption to the AF, it does not appear to cause  
 351 degeneration by eight weeks post injury.