**Supplementary Material**

**Methodology**

## S.M.1 Study description

### S.M.1.1 Samples description and preparation

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| **Table S1: Disc characteristics after dissection** | | | |
| Disc identity | Diameter: average and standard deviation (SD) (mm) | | Group |
| Tail 1 Disc 3 (T1D3) | 14.00.1 | 16.32.0 | Day0 |
| Tail 2 Disc 3 (T2D3) | 17.40.7 | Day0 |
| Tail 3 Disc 3 (T3D3) | 17.50.1 | Day0 |
| Tail 1 Disc 2 (T1D2) | 15.70.8 | 15.70.1 | Dynamic loading |
| Tail 2 Disc 4 (T2D4) | 15.80.3 | Dynamic loading |
| Tail 3 Disc 5 (T3D5) | 15.70.0 | Dynamic loading |
| Tail 1 Disc 1 (T1D1) | 16.50.6 | 15.90.9 | Active dynamic unloading |
| Tail 2 Disc 5 (T2D5) | 14.90.1 | Active dynamic unloading |
| Tail 3 Disc 4 (T3D4) | 16.30.3 | Active dynamic unloading |
| Tail 2 Disc 2 (T2D2) | 18.40.1 | 18.10.4 | Free swelling |
| Tail 3 Disc 2 (T3D2) | 17.80.2 | Free swelling |

### S.M.1.2 Equipment and consumables

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| **Table S2: Equipment and consumables** | | | |
| **Equipment** | | Penicillin-streptomycin (P/S) | Gibco #15140-122 |
| Bandsaw | Exakt 300 | Phosphate Buffered Saline Solution (PBS) | Sigma Aldrich #P4417 |
| Callipers | Mitutoyo, Absolute AOS Digimatic | Primocin | InvivoGen ant-pm-2 |
| Cryostat | Thermo Scientific, CryoStar NX70 | Resin (UV-sensitive) | Formlabs #BioMed Clear FLBMCL01 |
| Hammering device for pulverization | See Caprez et al. [58] | Sodium bicarbonate | Sigma Aldrich #S6014 |
| Jet lavage system | Zimmer Pulsavac #00-5150482-00 | Sodium pyruvate | Sigma #P5280 |
| Mechanical loading device with 250N load cell | Acumen III, MTS Systems Corporation |  |  |
| Microplate reader | Tecan, Infinite 200 Pro M Plex | **Analyses** | |
| Microscope | Olympus BX63 | 1-bromo, 3-chloropropane | ThermoScientific #106862500 |
| Quant Studio 6 instrument | Applied Biosystems | Chondroitin 4-sulfate sodium salt | Fluka BioChemika, #27042 |
| Tissue lyser (oscillating mill) | Retsch, MM400 | 1,9 Dimethy-methylene blue | Aldrich #341088 |
|  |  | Ethidium homodimer | Sigma-Aldrich #46043 (1mg/mL in DMSO) |
|  |  | Fast Green | Sigma #F7258; 1% |
|  |  | Gene Expression Master Mix (TaqMan) | ThermoScientific #4370074 |
| **Consumables** | | Lactate dehydrogenase | Roth #4421.4 |
| **Disc cultures** | | Methanol | Alcosuisse #16294-550 |
| Ascorbate 2-phosphate | Sigma #A8960 | Pronase | Roche #11459643001 (2mg/mL; 5mL/100mg tissue) |
| Dulbecco’s Modified Eagle Medium High Glucose (DMEM HG) | Gibco #52100-039 | Proteinase K | Roche #03115852001 (1mg/mL; 1mL proteinase K solution/10mg dry tissue) |
| Fetal Bovine Serum (FBS) | Corning #35-079-CV | RNeasy spin column | Qiagen RNeasy Mini #74106 |
| Hepes | Gibco #15630-056 | Safranin O | Sigma #S8884; 0.1% |
| ITS+Premix | Corning #354352 | SuperScript VILO cDNA Synthesis Kit | Invitrogen #11754 |
| Krebs-Ringer solution | BBraun #FREU924 | TRI Reagent™ | Molecular Research Center #TR118 |
| Minimum Essential Medium Non-Essential Amino Acids Solution (MEM NEAA) | Gibco #11140-035 | Weigert's Haematoxylin | Sigma #HT1079 |

Medium adapted from Dulbecco’s Modified Eagle Medium High Glucose

* DMEM HG 13.38g/L
* Sodium bicarbonate 3.7g/L
* Sodium pyruvate 0.11g/L
* + 1% P/S
* + additives
  + FBS 2%
  + Hepes 1%
  + MEM NEAA 1%
  + ITS+Premix 1%
  + Ascorbate 2-phosphate 0.1%
  + Primocin 0.1%

The pH of the medium after preparation was 7.4. The pH variation during the study was small (0.3), the pH of the collected medium oscillated between 7.1 and 7.7.

## S.M.2 Outcomes

### S.M.2.1 Viability cell counting workflow and validation

Manual cell counting for viability purposes takes time and is prone to intra- and interobserver errors, which undermines the reproducibility of the measurement and consequently the comparison of results within the field. Moreover, workflows with manual thresholding are not recommended due to reproducibility flaws. We propose a workflow that includes automatic thresholding and counting of the blue, blue/red (or purple) and red cells [80]. Table S3 below compares manual and automatic counting methods.

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| --- | --- | --- |
| **Table S3: Comparison manual and automatic cell counting workflow (lactate dehydrogenase and ethidium homodimer (LDH/Eth))** | | |
| Parameters | Manual counting | Automatic counting |
| Intra- and inter-observer variability | High | Inexistant |
| Microscope parameters | Variable | Fixed (possibility to compare different studies) |
| Format working file | Processed image (.jpeg) | Original image (.vsi) |
| Cell selection | Manual selection of single cells | Automatic thresholding |
| Time required | Time consuming | Time effective |

We divided the total area of the LDH/Eth sections into the three regions of the disc, defining the NP as the central 40% and the AFo as the outer 10% on both sides, with the AFi corresponding to the transition zone (10% on both sides) between the NP and the AFo, expressed by cell density and cell orientation. We counted the cells in several regions of interest (ROI) randomly placed within the three disc regions (4 in the NP, 3 or in the AFi or 4 if viability wasn't uniform and 2 in the AFo). Based on preliminary tests, the code applies the Otsu threshold for both brightfield and fluorescence images, includes the watershed function and excludes particles with an area smaller than 10μm2 and 3μm2, considered as artefacts, from the brightfield and fluorescence images, respectively.

We validated the developed workflow i.e. we manually counted the blue, blue/red (or purple) and red cells, then ran the code and determined the agreement between the two, manual and automatic, counting methods for cell viability and for cell density. The percentage of viable cells was calculated as the ratio of viable cells to the total number of cells (blue cells / (blue + red cells - purple cells)\*100). The number of cells per ROI (0.178mm2) was used to calculate cell density.

**Results**

## S.R.1 Biological evaluation

### S.R.1.1 Viability cell counting workflow and validation

The code gave correct results. In a few cases, the code gave erroneous results which were easy to detect, i.e. either viability was greater than 100% or the number of blue/red cells was greater than the number of blue cells. These errors are due to a small difference between the background and the cells leading to incorrect thresholding. The erroneous results were excluded from further analysis.

A graph with a red line

Description automatically generated

Figure S1: Agreement between the viability, expressed in %, obtained by the manual and the automatic counting methods for the different regions of the IVD (nucleus pulposus (NP): ICC=0.96 and n=41 pairs; inner annulus fibrosus (AFi): ICC=0.97 and n=34 pairs; outer annulus fibrosus (AFo): ICC=0.97 and n=20 pairs).

Figure S1 shows the agreement between the viability, expressed in %, obtained by the manual and the automatic counting methods for the different regions of the disc. The agreement, expressed as intraclass coefficient correlation (ICC; two-way mixed model and absolute agreement) is excellent in the three regions of the IVD (NP: ICC=0.96 and n=41 pairs; AFi: ICC=0.97 and n=34 pairs; AFo: ICC=0.97 and n=20 pairs)[81].

The ICC of the cell density results of both counting methods is excellent in the NP (ICC=0.94) and the AFi (ICC=0.98) and substantial in the AFo (ICC=0.74).

The automatic counting is 6 to 9x faster than the manual counting (5’ to 6’ vs 35’ to 45’ per section).

The results demonstrate that the automatic counting method is a valid time-effective method to compute the viability of LDH/Eth cryosections of bovine tail intervertebral discs.

### S.R.1.2 Morphology

A close up of a painting

Description automatically generated

Figure S2: Safranin O/Fast Green images (20x; scale bar 2mm) as illustration. Samples from Tail 1 of the dynamic loading group (a) and the active dynamic unloading group (b).

We did not observe differences between the groups.

## S.R.2 Mechanical evaluation

### S.R.2.1 Force-displacement curves

Dynamic loading

A graph of a number of numbers

Description automatically generated with medium confidence

Active dynamic unloading

A collage of images of a worm

Description automatically generated with medium confidenceFigure S3: Force-displacement curves of all the samples, of both groups, on the three loading days, at cycles 15 and 1439. Both parts of each curve are plotted in different colours. The area under the curve is represented by the grey area in between both parts of each curve. In the active dynamic unloading group, the neutral zone is delimited by the black dots on both parts of each curve. The scale is identical for both curves of one loading day but varies over loading days and samples to increase the visibility of the shape of the curves.

Note: We excluded the elastic zone 2 from the upper part (green part) of the curve of sample T2D5 on the second loading day at cycle 15 (T2D5 Unloading2 Cycle 15) from the analysis of the slope over time (Figure 12 of the Main manuscript) due to the very limited number of data points in that zone and the extremely low coefficient of determination (r2=0.07) indicative of an unrepresentative stiffness value.

### S.R.2.2 Displacement over time

A diagram of a graph

Description automatically generated with medium confidence

Figure S4: Maximum displacement over time of the different samples. Samples of the same tail, in both the dynamic loading and the active dynamic unloading groups, for the three loading days are represented in one graph. Each graph represents a different tail.

**References**

[80] Bankhead P. Analyzing fluorescence microscopy images with ImageJ. Nikon Imaging Center@Heidelberg University: Heidelberg University. 2010‒2012.

[81] Watson PF, Petrie A. Method agreement analysis: a review of correct methodology. Theriogenology. 2010; 73: 1167‒1179. https://doi.org/10.1016/j.theriogenology.2010.01.003.