

**Differential biomechanical properties of mouse distal colon and rectum
innervated by the splanchnic and pelvic afferents**

Saeed Siri*, Franz Maier#, Longtu Chen*, Stephany Santos#, David M. Pierce#,* ,

Bin Feng*

*Department of Biomedical Engineering, University of Connecticut, CT 06269

#Department of Mechanical Engineering, University of Connecticut, CT 06269

Abbreviated title: Biomechanics of mouse distal colon and rectum

Manuscript length: 18 pages, 8 figures, 0 table

Words: 236 for Abstract, 645 for Introduction, 1469 for Discussion

Conflict of interest: The authors claim no conflict of interests.

Acknowledgements: supported by NIH grant K01 DK100460 (BF) and NSF grants

CMMI-1727185 (BF and DP)

Correspondence:

Bin Feng, Ph.D.

Department of Biomedical Engineering, University of Connecticut

260 Glenbrook Road, Unit 3247, Storrs, CT 06269-3247, USA

E-mail: fengb@uconn.edu

Phone: 860-486-6435, fax: 860-486-2500

ABSTRACT

Visceral pain is one of the principal complaints of patients with Irritable Bowel Syndrome (IBS), and this pain is reliably evoked by mechanical distension/stretch of distal colon and rectum (colorectum). This study focuses on the biomechanics of the colorectum that could play critical roles in mechanical neural encoding. We harvested the distal 30 mm of the colorectum from mice, divided evenly into three 10-mm-long segments (colonic, intermediate and rectal), and conducted biaxial mechanical stretch tests and opening-angle measurements for each tissue segment. In addition, we determined the collagen fiber orientations and contents across the thickness of the colorectal wall by nonlinear imaging via second harmonic generation (SHG). Our results reveal a progressive increase in tissue compliance and pre-stress from colonic to rectal segments, which supports prior electrophysiological findings of distinct mechanical neural encodings by afferents in the lumbar splanchnic nerves (LSN) and pelvic nerves (PN) that dominate colonic and rectal innervations, respectively. The colorectum is significantly more viscoelastic in the circumferential direction than in the axial direction. In addition, our SHG results reveal a rich collagen network in the submucosa and orients approximately ± 30 degrees to the axial direction, consistent with the biaxial test results presenting almost twice the stiffness in axial direction versus the circumferential direction. Results from current biomechanical study strongly indicate the prominent roles of local tissue biomechanics in determining the differential mechanical neural encoding functions in different regions of the colorectum.

NEW & NOTEWORTHY

Mechanical distension/stretch – not heat, cutting or pinching – reliably evokes pain from distal colon and rectum. We report different local mechanics along the longitudinal length of the colorectum, which is consistent with the existing literature on distinct mechanotransduction of afferents innervating proximal and distal regions of the colorectum. This study draws attention to local mechanics as a potential determinant factor for mechanical neural encoding of the colorectum, which is crucial

in visceral nociception.

Keywords:

Bi axial test; visceral pain; irritable bowel syndromes; mechanotransduction; second harmonic generation

INTRODUCTION

Irritable bowel syndrome (IBS) is a frequent cause of patient visits to gastroenterologists with the principal complaint of prolonged visceral pain (12). Unlike other types of pain, visceral pain has a unique biomechanical component: it is mechanical distension/stretch of hollow visceral organs – not heat, cutting or pinching that reliably evokes pain from these organs (32). Accordingly, the distension of the distal colon and rectum (colorectum), i.e., colorectal distension is an effective means to cause visceral pain in both IBS patients and healthy volunteers (33, 50). In addition, heightened pain perception to rectal or colonic distension (visceral hypersensitivity) is considered a biomarker for IBS (5).

Colorectal mechanotransduction, i.e., encoding and transmission of mechanical stimuli to the colorectum to inform the central nervous system is undertaken by sensory innervations of the colorectum, which consist of sensory afferents in both lumbar splanchnic nerves (LSN) and pelvic nerves (PN). Both direct mechanical stretch of sensory nerve endings (7) and indirect effect from smooth muscle contraction (spasm) (40) can effectively activate mechanosensitive colorectal afferents. In the colorectum, the proximal portion (mostly colonic) is predominantly innervated by afferent endings in the LSN pathway whereas the distal portion (mostly rectal) is innervated by the PN pathway (2, 8). The mechanical neural encoding by colorectal afferents has been systematically characterized by us and others via single-unit recordings of action potentials from afferent nerve axons in the LSN and PN attached to the colorectum (7-11, 13, 14). In particular, we implemented an electrical stimulation protocol to unbiasedly identify all afferent endings in the colorectum, characterized their neural encoding functions, and classified them based on response, or lack thereof, to three distinct mechanical stimuli applied to the

colorectum: punctate probing, mucosal stroking and circumferential stretching (8). Together, mechanosensitive afferent classes contribute to over 67% and 77% of the sensory innervation in the LSN and PN pathways, respectively; mechanically-insensitive afferents (MIA) contribute the rest (8). In diseased condition of prolonged visceral hypersensitivity, ~70% MIAs in the PN pathway and ~20% in the LSN pathway became sensitized and acquire mechanosensitivity to generate *de novo* input to the central nervous system (8, 13, 14, 23). In addition to the different innervation regions in the colorectum, the LSN and PN afferents also have different neural encoding profiles to mechanical stimuli: the neural encoding of circumferential colorectal stretch and mucosal stroking is mainly undertaken by the PN afferents while most LSN afferents encode punctate probing (8, 12). Also, a nerve lesion study indicates that a functioning PN, not LSN pathway is necessary for evoking behavioral nociceptive responses in mice *via* noxious colorectal distension (22).

Colorectal mechanotransduction occurs at afferent nerve endings embedded in the colorectal wall and relies on a biomechanical process, namely transmission of bulk mechanical deformation (e.g., colorectal distension) to local stress/strain distributions in microns-thick afferent nerve endings that triggers generation of action potentials at the spike initiation zone (15). Thus, knowledge about the biomechanics of distal colon and rectum is crucial in understanding colorectal mechanotransduction, however such knowledge is scarce in contrast to the available neurophysiological data. So far, no studies have attempted to contrast the biomechanical properties of distal colon (predominantly innervated by the LSN) and rectum (predominantly innervated by the PN). In the present study, we systematically conducted biaxial tissue testing on harvested mouse intestinal tissues of 7x7 mm² and discovered significant differences in tissue biomechanical properties along the axial length of the mouse colorectum. In addition, colorectal tissue showed significant anisotropy between the axial and circumferential directions. We also quantified residual stresses in the colorectum by measuring opening angles and recorded gradually increased residual stress from colonic to rectal regions.

In addition, we imaged with second harmonic generation (SHG) to visualize through-thickness fiber orientations and discovered a rich network of collagen fibers in the submucosal regions of the colorectum, a new anatomic evidence to support submucosa as a load carrying structure for large intestine.

MATERIALS AND METHODS

All experiments were reviewed and approved by the University of Connecticut Institutional Animal Care and Use Committee.

Colorectal tissue harvesting

Twenty one mice of 11 males and 10 females were used in this study (C57BL/6, Taconic, Germantown, NY), aged 8-12 weeks and weighing 20-30 g. All the mice used in this study were raised in individually ventilated cages (up to 5 mice per cage), bedded with aspen sani-chip bedding, fed with irradiated Teklad global diet and provided with light from 7 am to 9 pm. On an experimental day, one mouse was transferred to a fume hood in the morning for anesthesia by isoflurane inhalation, euthanized by exsanguination after perforating the right atrium, and transcordially perfused with oxygenated Krebs solution (in mM: 117.9 NaCl, 4.7 KCl, 25 NaHCO₃, 1.3 NaH₂PO₄, 1.2 MgSO₄·7H₂O, 2.5 CaCl₂, 11.1 D-Glucose, 2 butyrate, and 20 acetate) bubbled with carbogen (95% O₂, 5% CO₂). A midline laparotomy was performed and the pubic symphysis was transected to expose the pelvic floor organs. The distal 30 mm of the large bowel including the distal colon and rectum was dissected free of connective tissues and transferred to modified Krebs solution to which was added nifedipine (4 μM; L-type calcium channel antagonist to block muscle activities), penicillin-streptomycin (100 U/ml), and protease inhibitors (P2714, Sigma Aldrich, St. Louis, MO). To be consistent with prior electrophysiological and behavioral studies that implemented colorectal distension (e.g., (43)), the tissue was cannulated and distended by phosphate buffered saline at ascending levels of graded intraluminal pressure: 15, 30, 45, 60 mmHg, 10 sec for each distension. Graded distension was performed at least 4 times on each colorectum in modified Krebs solution at room temperature.

Opening angle measurements

Assuming the anus as zero coordinate, the 30 mm colorectum was divided into three even segments: 0-10 mm (rectal), 10-20 mm (intermediate), and 20-30 mm (colonic) as shown in **Fig. 1**, cf. (7). After performing the graded distension, one tissue ring of 2 mm length was collected from the proximal end of each segment. A photograph was taken of the cross-section of the rings (the no-load state). Then, each ring was cut radially under a stereomicroscope (M165, Leica Microsystems, DE), which may open into a sector to release residual stresses. Following a prior report (18), we allowed a 30-min period for complete release of the residual stress before taking another photograph (the zero-stress state). The opening angle is defined as the angle subtended by two radii drawn from the midpoint of the inner wall to the inner tips of two ends of the sector.

Biaxial stretch test and local strain measurement

All three segments of colorectum were cut open one by one along the mesentery, pinned flat, and kept in the Krebs solution for at least 15 minutes before performing the experiments. Three square-shaped specimens (approx. $7 \times 7 \text{ mm}^2$) were harvested from the colonic, intermediate and rectal segments as illustrated in **Fig. 1**, respectively. Each specimen was then mounted onto a biaxial tensile testing device via a custom-built adaptor consisting of long and narrow cantilevers to freely permit lateral deformations during the biaxial tests as shown in **Fig. 2A**. The 3-D-printed cantilever was made from acrylic resin (**Fig. 2B**, 0.1 mm resolution, Shapeways, New York City, NY) to enforce minimal lateral force during deformation (0.5 mN/mm). We aligned specimens in the circumferential and axial directions and tested them using a computer-controlled force/displacement actuator (Model 300D, Aurora Scientific, CA). Throughout the experiments, the tissue remained submerged in the ~120 mL of aforementioned modified Krebs solution at room temperature containing nifedipine, penicillin-streptomycin, and protease inhibitor. Consistent with the electrophysiological studies, we focused on quantifying the quasi-static material properties of the specimens by applying slow ramped force (0-80 mN) and

continuously recorded displacements in both circumferential and longitudinal directions. In a preliminary experiment, we determined a loading rate of 1.2 mN/sec as sufficient for providing repeatable and consistent force-displacement measurements (**Fig. 3**). Thus, the test protocol for each specimen consisted of 30 cycles of quasi-static ramped loading (0 – 80 mN) and ramped unloading (80 – 0 mN) at 1.2 mN/sec. From our preliminary study, the force-displacement response started to become repeatable after over 25 loading cycles. Thus, the first 27 cycles are for tissue pre-conditioning and the data from the last 3 cycles were averaged as the steady-state force-displacement response of the specimen. After completing the test, the specimen thickness was measured using a caliper under stereomicroscopy.

We recorded the biaxial extension of a subset of specimens with a stereo camera system (Q-400 μ DIC, Dantec Dynamics, Skovlunde, DK) and used commercially available software Istra4D (V4.4.3.414, Dantec Dynamics) for digital-image correlation (DIC) to obtain the 2-D deformation field. To track the deformation, we airbrushed (CM-C Plus, IWATA, Yokohama, JP) carbon dots ($< 2 \mu\text{m}$) on the specimen, with the serosal side facing the camera. We then calculated the local circumferential, axial and shear strain distribution throughout the specimen to verify homogenous deformations.

Imaging collagen fibers by second harmonic generation (SHG)

After mechanical testing, specimens were stretched biaxially to 80 mN, fixed with 4% paraformaldehyde for 60 min, and mounted onto a glass slide (Permunt, Fisher Scientific, Hampton, NH), with the serosa side facing the cover slip (No 1.5), for non-linear two-photon imaging of second harmonic generation. For imaging we used a LSM 780 (Carl Zeiss, Oberkochen, DE) with a 40x objective (C-Apochromat 40x/1.2 W Corr) with a working distance of 280 μm . We used a tunable two-photon light source (Chameleon, Coherent, Santa Clara, CA) to excite the SHG at 900 nm and collected the signal at 450 nm. This setup allowed label free imaging, highly specific to collagen fibers, through the entire thickness of our specimens (cf. (35)).

Histological staining of colorectum

After the completion of the mechanical stretch protocols, the 7x7mm colorectal patches were fixed with 4% paraformaldehyde for 60 min, embedded in paraffin and sectioned on a microtome at 8 μ m. Following staining with hematoxylin and eosin, the tissue was examined on an Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan) provided with appropriate filters and a Hamamatsu ORCA-ER, C4742-80 digital camera, using Hamamatsu Photonics Wasabi 150 software (Hamamatsu Photonics K.K., System Division, Hamamatsu City, Japan).

Data analysis

Data processing was conducted blind to the experimental conditions. For biaxial stretch tests, abrupt change of stretch force or displacement more than 10% of the peak value within 0.5 sec was considered as a sign of tissue tearing and the data was excluded from the analysis. We assumed a cylindrical coordinate (r, θ, z) for the tubular colorectum in radial, circumferential, and axial directions, respectively. We denoted T as the mean thickness in the unloaded reference configuration measured by the caliper. The measured lengths of the unloaded specimens were denoted as L_θ and L_z in the circumferential and axial directions, respectively. The biaxial stretch forces were denoted as f_θ and f_z . The stretch ratios ($\lambda_\theta, \lambda_z$) were calculated using the specimen geometry after the 27 cycles of the pre-condition as the reference coordinates (X_θ, X_z) normalized to the deformed coordinates (x_θ, x_z): $\lambda_\theta = x_\theta/X_\theta$ and $\lambda_z = x_z/X_z$. We then computed Cauchy stresses (σ) assuming incompressibility and negligible shear using $\sigma_{\theta\theta} = \lambda_\theta f_\theta / TL_z$, $\sigma_{zz} = \lambda_z f_z / TL_\theta$. Data were presented throughout as mean $\pm$ SEM unless specifically noted. One-way and two-way analyses of variance (ANOVA) or repeated-measures were performed as appropriate using SigmaPlot v9.0 (Systat software, Inc., San Jose, CA). Bonferroni post-hoc multiple comparisons were performed when F values for main effects were significant. Differences were considered significant when $p < 0.05$ (denoted by *). Before data collection, the sample sizes for the experiments were determined by estimated power analysis using standard deviations from prior tissue biomechanical studies by us and others. The statistical power of the study was further validated after the data collection to be

greater than 0.8.

RESULTS

Differential stress-strain behaviors from colonic to rectal region.

From the biaxial tissue testing results of force-displacement data, the Cauchy stress - stretch ratio relations from one example specimen were calculated and displayed in **Fig. 4A**. The ramped loading and unloading cycles were repeated 30 times for each specimen and the last three cycles were averaged as the test results. The 27 cycles of pre-conditioning stretch were plotted as grey curves indicating viscous creep during the tissue pre-conditioning. The loading portion of the Cauchy stress – stretch ratio relations from 21 colorectums were averaged and plotted in **Fig. 4B** for males and **4C** for females. Specimens showing apparent tissue tearing and/or abrupt change in the force-displacement curve were excluded, including specimens from 5 colonic (3 males and 2 females), 1 intermediate (male) and 3 rectal (2 males and 1 female) segments. There were no sex differences between the stress-strain plots in **Fig. 4B** and **4C** (Two-way repeated measure ANOVA, $F_{1,14} = 0.02$, $p = 0.89$ for colonic circumferential; $F_{1,14} = 0.002$, $p = 0.91$ for colonic axial; $F_{1,18} = 0.11$, $p = 0.75$ for intermediate circumferential; $F_{1,18} = 0.41$, $p = 0.53$ for intermediate axial; $F_{1,16} = 0.29$, $p = 0.6$ for rectal circumferential; $F_{1,16} = 0.51$, $p = 0.49$ for rectal axial). Thus, the data from both sexes were pooled together for subsequent analysis. The axial stress-strain properties showed significant differences between all three segments (Two-way ANOVA, $F_{2, 2195} = 282.5$, $p < 0.001$, post-hoc comparison, $p < 0.001$ for rectal vs. intermediated, rectal vs. colonic, and intermediate vs. colonic). The same significant difference was observed also in the circumferential stress-strain properties (Two-way ANOVA, $F_{2, 2117} = 225.3$, $p < 0.001$, post-hoc comparison, $p < 0.001$ for rectal vs. intermediated, rectal vs. colonic, and intermediate vs. colonic). Collectively, the distal colorectum showed a progressive increase in compliance from proximal to distal segments in both axial and circumferential directions. In addition, specimens from all 3 segments showed significant anisotropy between the circumferential and axial directions (Two-way ANOVA, $F_{1,30} = 43.9$, $p < 0.001$ for colonic; $F_{1,38} = 68.9$, $p < 0.001$ for intermediate; $F_{1,34} = 105.3$, $p < 0.001$ for rectal segments).

Differential viscoelastic behaviors between axial and circumferential directions

As illustrated in **Fig. 5A**, the viscoelastic behaviors of the specimen were quantified from the force-displacement relations as the area between the loading and unloading curves normalized by the total area under the loading curve, indicating the fraction of energy dissipation during the symmetric loading-unloading cycle. Displayed in **Fig. 5B** are the average fraction of energy loss from colonic, intermediate, and rectal segments in axial and circumferential directions, respectively. The fraction of energy dissipation is significantly higher in the circumferential direction than the axial one (Two-way ANOVA, $F_{1,102} = 102.4$, $p < 0.001$), indicating significant viscous properties in the circumferential direction. In contrast, the colorectum is comparatively elastic in the axial direction with minimal viscous dissipation (less than 5% energy dissipation). The fraction of energy dissipation is not different across different segments ($F_{1,2} = 0.63$, $p = 0.53$)

Increased residual stress from colonic to rectal region

The opening angles were measured from 2-mm-thick tissue rings harvested from the colorectums with representative photos shown in **Fig. 6A**. Since, no significant sex difference was detected in the above biomechanical tests, opening angles measured from both sexes were pooled together. Opening angles from seven colorectums were averaged and displayed in **Fig. 6B**, showing significantly higher opening angle in the rectal segments than in the colonic and intermediate segments (One-way ANVOV, $F_{2,18} = 134$, $p < 0.05$, post-hoc comparison, $p < 0.001$ for rectal vs. colonic and rectal vs. intermediate). In addition, the opening angle at the intermediate segment is significantly higher than at the colonic segments (post-hoc comparison, $p < 0.02$), collectively showing a trend of increased opening angle from proximal to distal direction.

Homogeneous deformation during biaxial stretch

Representative specimen in **Fig.7** shows average shear strain (-0.7480 ± 1.015 ,

mean $\pm$ STD) one order of magnitude smaller than average axial (5.010 ± 0.6725) and circumferential (9.041 ± 1.070) strains on the planar surface of a specimen during biaxial stretch. Further, axial and circumferential strains confirm reasonably homogeneous deformations at full extension with a STD approximately 10% of the mean.

Qualitative collagen fiber orientation through the colorectal wall

The integrity of the colorectal tissue patches after the mechanical biaxial stretch protocol was confirmed by the H&E staining in **Fig. 8A**, which showed no apparent tissue tearing or damage in either colonic, intermediate or rectal segments. SHG imaging allowed us to resolve the individual layers through the thickness of the tissue, with representative images shown in **Fig. 8B**. The colorectal wall consists of the following layers from the external serosal side to the internal mucosal side: serosal (S), longitudinal muscular (LM), intermuscular (IM), circular muscular (CM), submucosal (SM), and mucosal (M) layers, each with a distinct collagen fiber orientations. Collagen fibers in the muscular layers (LM and CM) align with the direction of the muscle fibers. The S layer shows thicker fibers, aligned in axial direction and the IM contains thin, isotropic fibers. The SM layer shows thick fiber bundles aligned about ± 30 degrees to the axial direction. Fibers in M concentrate in the wall of the crypts.

DISCUSSION

This is the first study to compare and contrast the colorectal regions with dominant innervations by the LSN (colonic segment) and PN (rectal segment), respectively. A handful of previous studies on the biomechanics of large intestine were conducted on colon segments proximal to the LSN and PN innervation (3, 4), and usually neglected the micro-scale mechanics (18, 36, 37). Biomechanics of more proximal portions of the gastrointestinal (GI) tract have been more extensively studied, including the small intestine (16, 27, 42, 47-49) and esophagus (25, 26, 28, 39, 44, 45). However, both the physiological function and anatomic structure of the distal colorectum differ significantly from their proximal counterparts in the GI tract (24), preventing direct translation of knowledge to the distal colon and rectum. Since the LSN and PN innervations in mice are clustered in short segments of the colorectum (~10mm), we were prohibited from using the conventional approach of inflating the tubular colorectum to determine the stress-strain relationship as used previously on longer intestinal tissues, e.g., rat large (18) and small intestines (6) of more than 30 mm in length. To address that, we developed this novel biaxial testing setup and determined the stress-strain behaviors from small square specimens (7x7 mm) harvested from different regions in the colorectum. We implemented a force actuator with force precision of 0.1 mN and displacement precision of 0.1 mm (Model 300D, Aurora Scientific, Aurora, CA) to deliver the biaxial stretch force. We did not attempt to study specimens smaller than 7x7mm, which will require more accurate recordings of stretch force and displacement beyond the capacity of our current equipment. To allow free lateral movement of the tissue, we developed custom-built adaptors consisting of 30 mm-long cantilevers for mounting the tissue. The long cantilevers were designed and verified to have a low elastic modulus laterally (0.5 mN/mm) to allow minimal resistance to the lateral deformation of the tissue. The stress – stretch ratio properties determined from our biaxial device is qualitatively comparable to the stress – strain data reported previously from rat large intestine (18, 46). In addition, our local strain measurements via speckle tracking of the sprayed carbon dots

validates homogeneous biaxial deformations in our testing (cf. **Fig.7**). The limitation of our biaxial tests is the stress concentration at the specimen mounting sites on the cantilever tips (by minute metal pins) that causes the specimen to fail at the mounting sites before reaching the rupture stress of the colorectum. However, the maximum stretch force of 80 mN implemented throughout this study correlates to ~40 mmHg intraluminal colorectal pressure, which covers most of the physiological pressure range of mouse colorectum and is beyond the noxious threshold of 20 mmHg for mice (7, 21). Thus, our novel approach of biaxial mechanical tissue testing adequately determines the local physiological stress – strain properties of small tissue samples like mouse colorectum with typical circumference of less than 10 mm.

Our biaxial tests reveal apparent tissue anisotropy in mouse colorectum with stiffer stress-strain curves in the axial direction than the circumferential direction, consistent with prior studies on small and large intestines (3, 18, 34, 46). Biological tissues generally show some anisotropy, such as esophageal (39), myocardium (38), and aorta (29), which likely results from the distributions and orientations of collagen fibers, the load-carrying and reinforcing proteins in soft tissues (19). The colorectum is about twice as stiff in the axial direction than the circumferential direction. Our SHG images suggest thick axially aligned collagen fibers in the submucosa layer, consistent with prior findings via electron microscopy in rat small intestine (17, 30, 31). The serosal layer as a connective tissue membrane is also rich in collagen but its contribution to the bulk colorectal mechanical strength is limited by its thinness. It might have a similar protective role to prevent overstretching like the outermost adventitia layer of the arterial wall (20). Our findings of a collagen-rich network in mouse colorectal submucosa together with similar findings by others in rat small intestine collectively suggest that the submucosa is the load carrying skeleton for the gastrointestinal tract (17, 30, 31). In addition, prior electron microscopy studies in rat small intestine determined that the collagen fibers are oriented ± 30 degrees to the axial direction (31), in good agreement with our current findings that colorectum is almost twice as stiff in the axial direction than in the circumferential direction.

Submucosa as the skeleton of the gut indicates its protective role in high-intensity colorectal distension and probing, which logically suggests the presence of nociceptive afferent endings in the submucosa for detecting noxious and tissue-injurious mechanical stimuli. This is further supported by a neural afferent tracing study which showed the highest concentration of nerve endings in the submucosa (32% of total afferent endings), even higher than in the myenteric ganglion (22%) and circular muscle layer (25%) (41).

Prior studies of colorectal mechanotransduction have largely focused on the neural encoding function of sensory afferents via electrophysiological recordings from lumbar splanchnic (LSN) and pelvic nerves (PN), two major innervation pathways of the distal colon and rectum (1, 2, 8). The LSN and PN innervations appear to dominate in the proximal colonic and distal rectal regions of the colorectum, respectively (7). We need to emphasize that there is no clear separation of the LSN and PN innervation regions in the colorectum, as anterograde tracing studies have shown that some PN afferents can traverse considerable distances to the proximal region generally innervated by the LSN afferents (41). The biomechanics of the colorectal wall could play critical roles in the initiation of mechanotransduction. Consistent with the different neural encoding characteristics between the LSN and PN pathways, we discover the apparent difference in biomechanical properties between the colonic and rectal segments in 1) tissue stiffness, 2) viscoelasticity, 3) residual stress, and 4) anatomic thickness. The circumferential stiffness is significantly lower in the rectum than in the colon, which is consistent with the dominant PN innervation of the rectum with the majority of the afferents responding to circumferential colorectal stretch (8, 12). LSN afferents that predominately innervate the colonic region generally do not respond to circumferential colorectal stretch. The lower rectal stiffness in circumferential direction also agrees with electrophysiological findings that PN afferents with receptive fields in the rectal region have higher firing rates to circumferential stretch than afferents in the colonic region (7). The colorectum tissue is viscoelastic and dissipates more energy under deformation in the circumferential

direction than in the axial, which could underlie the adaptation of afferent activities to circumferential colorectal stretch (7, 8). The range of opening angles in this report is consistent with a previous study on mouse large intestine (18). Unlike the small intestine that folds towards the out surface when cut open (6), the opening angle of the colorectum is comparatively small, indicating modest compression of the inner mucosa layer and extension of the out muscular layers in the physiological condition to mitigate stress concentrations in the inner layer during intraluminal distension. The increasing opening angle towards the rectum indicates that the rectal afferents in the muscular layers are pre-stretched in the physiological conditions, which might contribute to their higher firing rate than afferents in the colonic segments (7). Last, the rectum is significantly thicker than the colon (7), which implies more severe stress concentration in the inner layer of the rectum during colorectal distension than in the colon. This is also consistent with the current report of larger opening angle (i.e., higher pre-stress) in the rectum than in the colon to attenuate stress concentration in the inner layer.

In summary, we systematically conducted mechanical tissue testing on the distal 30 mm of mouse large intestine, i.e., the colorectum consisting of colonic, intermediate, and rectal segments that are differentially innervated by sensory afferents in the LSN and PN. Biaxial stretch testing reveals that the colorectum is almost twice as stiff in the axial direction than in the circumferential direction, consistent with the finding of a rich network of collagen fibers in the submucosa that are aligned about ± 30 degrees to the axial direction. The rectal region is more compliant than the proximal region in the circumferential direction. The colorectum is significantly more viscoelastic in the circumferential direction than in axial direction. The rectum is thicker than the proximal regions and subjected to more pre-compression in the mucosa and pre-tension in the muscular layers, which could contribute to the higher firing rate of afferents with endings in the rectal muscular layers. The current study reveals the distinct mechanical properties between the colonic and rectal regions in the colorectum, consistent with the differential neural

encoding functions of LSN and PN afferents that dominate the colonic and rectal innervations, respectively. Further biomechanical studies of the colorectum are required to complement the existing electrophysiological findings to synergistically advance our mechanistic understanding of colorectal mechanotransduction, which is crucial in IBS-related visceral pain and hyperalgesia.

FIGURE LEGENDS

Figure 1. The schematic of the sensory innervations of mouse distal colon and rectum (colorectum) by lumbar splanchnic and pelvic nerves. Considering the anus as zero coordinate, the distal 30 mm of the colorectum is divided into rectal (0 - 10 mm), intermediate (10 – 20 mm) and colonic (20 - 30 mm) segments.

Figure 2. Biaxial tissue testing of specimen from mouse colorectum and local strain measurement. (A) The specimen (7x7 mm² squares) were harvested from three segments of the colorectum (rectal, intermediate, and colonic) and mounted onto a custom-built adaptor for biaxial stretch tests. (B) The adaptor fabricated by 3-D printing was further trimmed and configured as in (A) to allow lateral displacement of the specimen during biaxial stretch with minimal resisting force (0.5 mN/mm). (C) Photo images of a specimen sprayed with carbon dots for local strain measurement by optical tracking. (D) Magnified view of the carbon dots in the white square region in (C), showing speckle patterns of microns in size.

Figure 3. Cauchy stress – stretch ratio results from quasi-static biaxial stretch test at multiple stretch rates. Linear ramped loading (0 – 80 mN) and unloading (80 – 0 mN) forces were applied to the specimen at multiple rates: 0.4, 1.2, and 2 mN/sec. Stretch rates below 1.2 mN/sec provide repeatable and robust results and ramped forces of 1.2 mN/sec were used as the testing protocol throughout.

Figure 4. Colorectal stress – strain behavioral determined by biaxial tissue stretch tests. (A) Representative Cauchy stress – stretch ratio curves (solid colored lines) calculated as the average of the last three loading/unloading cycles of the total 30 cycles. Displayed in grey lines were the first 27 loading/unloading cycles for tissue pre-conditioning. The Cauchy stress – stretch ratio curves in the loading cycle at colonic, intermediate and rectal segments from male (B) and female (C) mice, respectively. Repeatable stretch forces were delivered to all specimens and the average stretch ratios were plotted along with the standard error mean. For male colorectum, data from 8 colonic, 10 intermediate, and 9 rectal specimens were analyzed. For female colorectum, data from 8 colonic, 10 intermediate, and 9 rectal

specimens were analyzed.

Figure 5. Colorectal tissue viscoelasticity quantified as fraction of energy loss. (A) The force-displacement curves were used to calculate the fraction of energy loss using the grey area enclosed by the loading and unloading curves normalized by the total area under the loading curve (grey plus hatched area). (B) Fraction of energy loss from 16 colonic, 20 intermediate and 18 rectal segments. The horizontal bars indicate the average fraction of energy loss.

Figure 6. Residual stresses in the colorectum quantified by opening angle measurements. (A) Photographs of tissue rings (2 mm thick) harvested from rectal, intermediate, and colonic segments before and after cutting open. The opening angles are labeled as θ . (B) Average opening angles measured from seven colons are 42.4° , 58.4° and 119.4° , gradually increasing from proximal to distal locations in the colorectum.

Figure 7. Planer axial, circumferential and shear strain distribution during biaxial tissue testing on the $7 \times 7 \text{ mm}^2$ colorectal specimen verifies homogeneous strain distribution. Average shear strain is one order of magnitude lower than strain in axial and circumferential directions.

Figure 8. Histological studies by H & E staining (A) and SHG imaging (B), respectively. The H & E staining confirms the tissue integrity post the biaxial mechanical stretch protocol. Representative SHG images show different through-thickness fiber orientations and thicknesses for the corresponding layers. C. muscular: circular muscular; L. muscular: longitudinal muscular. Scale bars indicate $200 \mu\text{m}$ in (A) and $50 \mu\text{m}$ in (B).

REFERENCES

1. **Brierley SM, Carter R, Jones W, III, Xu L, Robinson DR, Hicks GA, Gebhart GF, and Blackshaw LA.** Differential chemosensory function and receptor expression of splanchnic and pelvic colonic afferents in mice. *Journal of Physiology* 567: 267-281, 2005.
2. **Brierley SM, Jones RC, 3rd, Gebhart GF, and Blackshaw LA.** Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. *Gastroenterology* 127: 166-178, 2004.
3. **Carniel E, Gramigna V, Fontanella C, Frigo A, Stefanini C, Rubini A, and Natali A.** Characterization of the anisotropic mechanical behaviour of colonic tissues: experimental activity and constitutive formulation. *Experimental physiology* 99: 759-771, 2014.
4. **Carniel EL, Gramigna V, Fontanella CG, Stefanini C, and Natali AN.** Constitutive formulations for the mechanical investigation of colonic tissues. *Journal of biomedical materials research Part A* 102: 1243-1254, 2014.
5. **Clarke G, Quigley EM, Cryan JF, and Dinan TG.** Irritable bowel syndrome: towards biomarker identification. *Trends Mol Med* 15: 478-489, 2009.
6. **Dou Y, Fan Y, Zhao J, and Gregersen H.** Longitudinal residual strain and stress-strain relationship in rat small intestine. *BiomedEng Online* 5: 37, 2006.
7. **Feng B, Brumovsky PR, and Gebhart GF.** Differential roles of stretch-sensitive pelvic nerve afferents innervating mouse distal colon and rectum. *American journal of physiology Gastrointestinal and liver physiology* 298: G402-409, 2010.
8. **Feng B and Gebhart GF.** Characterization of silent afferents in the pelvic and splanchnic innervations of the mouse colorectum. *American journal of physiology Gastrointestinal and liver physiology* 300: G170-180, 2011.
9. **Feng B and Gebhart GF.** In vitro functional characterization of mouse colorectal afferent endings. *Journal of visualized experiments : JoVE*: 52310, 2015.
10. **Feng B, Joyce SC, and Gebhart GF.** Optogenetic activation of mechanically insensitive afferents in mouse colorectum reveals chemosensitivity. *American journal of physiology Gastrointestinal and liver physiology* 310: G790-798, 2016.
11. **Feng B, Kiyatkin ME, La JH, Ge P, Solinga R, Silos-Santiago I, and Gebhart GF.** Activation of guanylate cyclase-C attenuates stretch responses and sensitization of mouse colorectal afferents. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 33: 9831-9839, 2013.
12. **Feng B, La JH, Schwartz ES, and Gebhart GF.** Irritable bowel syndrome: methods, mechanisms, and pathophysiology. Neural and neuro-immune mechanisms of visceral hypersensitivity in irritable bowel syndrome. *American journal of physiology Gastrointestinal and liver physiology* 302: G1085-1098, 2012.
13. **Feng B, La JH, Schwartz ES, Tanaka T, McMurray TP, and Gebhart GF.** Long-term sensitization of mechanosensitive and -insensitive afferents in mice with

persistent colorectal hypersensitivity. *American journal of physiology Gastrointestinal and liver physiology* 302: G676-683, 2012.

14. **Feng B, La JH, Tanaka T, Schwartz ES, McMurray TP, and Gebhart GF.** Altered colorectal afferent function associated with TNBS-induced visceral hypersensitivity in mice. *American journal of physiology Gastrointestinal and liver physiology* 303: G817-824, 2012.

15. **Feng B, Zhu Y, La JH, Wills ZP, and Gebhart GF.** Experimental and computational evidence for an essential role of NaV1.6 in spike initiation at stretch-sensitive colorectal afferent endings. *Journal of neurophysiology* 113: 2618-2634, 2015.

16. **Frokjaer JB, Andersen SD, Drewes AM, and Gregersen H.** Ultrasound-determined geometric and biomechanical properties of the human duodenum. *Digestive diseases and sciences* 51: 1662-1669, 2006.

17. **Gabella G.** The collagen fibrils in the collapsed and the chronically stretched intestinal wall. *Journal of ultrastructure research* 85: 127-138, 1983.

18. **Gao C and Gregersen H.** Biomechanical and morphological properties in rat large intestine. *JBiomech* 33: 1089-1097, 2000.

19. **Holzapfel GA.** Biomechanics of soft tissue. *The handbook of materials behavior models* 3: 1049-1063, 2001.

20. **Holzapfel GA, Gasser TC, and Ogden RW.** A New Constitutive Framework for Arterial Wall Mechanics and a Comparative Study of Material Models. *Journal of elasticity and the physical science of solids* 61: 1-48, 2000.

21. **Kamp EH, Jones RC, III, Tillman SR, and Gebhart GF.** Quantitative assessment and characterization of visceral nociception and hyperalgesia in mice. *AmJPhysiol GastrointestLiver Physiol* 284: G434-G444, 2003.

22. **Kyloh M, Nicholas S, Zagorodnyuk VP, Brookes SJ, and Spencer NJ.** Identification of the visceral pain pathway activated by noxious colorectal distension in mice. *Front Neurosci* 5: 16, 2011.

23. **La JH, Feng B, Schwartz ES, Brumovsky PR, and Gebhart GF.** Luminal hypertonicity and acidity modulate colorectal afferents and induce persistent visceral hypersensitivity. *American journal of physiology Gastrointestinal and liver physiology* 303: G802-809, 2012.

24. **Leung PS, editor.** *The gastrointestinal system : gastrointestinal, nutritional, and hepatobiliary physiology.* Netherlands: Dordrecht : Springer, 2014.

25. **Liao D, Fan Y, Zeng Y, and Gregersen H.** Stress distribution in the layered wall of the rat oesophagus. *Medical engineering & physics* 25: 731-738, 2003.

26. **Liao D, Zhao J, Fan Y, and Gregersen H.** Two-layered quasi-3D finite element model of the oesophagus. *Medical engineering & physics* 26: 535-543, 2004.

27. **Lu X, Zhao J, and Gregersen H.** Small intestinal morphometric and biomechanical changes during physiological growth in rats. *Journal of biomechanics* 38: 417-426, 2005.

28. **Natali AN, Carniel EL, and Gregersen H.** Biomechanical behaviour of oesophageal tissues: material and structural configuration, experimental data and constitutive analysis. *Medical engineering & physics* 31: 1056-1062, 2009.

29. **Niestrawska JA, Viertler C, Regitnig P, Cohnert TU, Sommer G, and Holzapfel GA.** Microstructure and mechanics of healthy and aneurysmatic abdominal aortas: experimental analysis and modelling. *Journal of The Royal Society Interface* 13: 20160620, 2016.
30. **Orberg J, Baer E, and Hiltner A.** Organization of collagen fibers in the intestine. *Connective tissue research* 11: 285-297, 1983.
31. **Orberg J, Klein L, and Hiltner A.** Scanning electron microscopy of collagen fibers in intestine. *Connective tissue research* 9: 187-193, 1982.
32. **Pasricha PJ, Willis WD, and Gebhart GF.** *Chronic abdominal and visceral pain: theory and practice*: Taylor & Francis US, 2006.
33. **Price DD, Zhou Q, Moshiree B, Robinson ME, and Verne GN.** Peripheral and central contributions to hyperalgesia in irritable bowel syndrome. *The journal of pain : official journal of the American Pain Society* 7: 529-535, 2006.
34. **Sacks MS and Gloeckner DC.** Quantification of the fiber architecture and biaxial mechanical behavior of porcine intestinal submucosa. *Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials* 46: 1-10, 1999.
35. **Schriebl AJ, Zeindlinger G, Pierce DM, Regitnig P, and Holzapfel GA.** Determination of the layer-specific distributed collagen fibre orientations in human thoracic and abdominal aortas and common iliac arteries. *Journal of the Royal Society, Interface / the Royal Society* 9: 1275-1286, 2012.
36. **Sokolis DP, Orfanidis IK, and Peroulis M.** Biomechanical testing and material characterization for the rat large intestine: regional dependence of material parameters. *Physiological measurement* 32: 1969-1982, 2011.
37. **Sokolis DP and Sassani SG.** Microstructure-based constitutive modeling for the large intestine validated by histological observations. *Journal of the mechanical behavior of biomedical materials* 21: 149-166, 2013.
38. **Sommer G, Haspinger DC, Andrä M, Sacherer M, Viertler C, Regitnig P, and Holzapfel GA.** Quantification of shear deformations and corresponding stresses in the biaxially tested human myocardium. *Annals of biomedical engineering* 43: 2334-2348, 2015.
39. **Sommer G, Schriebl A, Zeindlinger G, Katzensteiner A, Ainodhofer H, Saxena A, and Holzapfel GA.** Multiaxial mechanical response and constitutive modeling of esophageal tissues: Impact on esophageal tissue engineering. *Acta biomaterialia* 9: 9379-9391, 2013.
40. **Spencer NJ, Kerrin A, Zagorodnyuk VP, Hennig GW, Muto M, Brookes SJ, and McDonnell O.** Identification of functional intramuscular rectal mechanoreceptors in aganglionic rectal smooth muscle from piebald lethal mice. *American journal of physiology Gastrointestinal and liver physiology* 294: G855-G867, 2008.
41. **Spencer NJ, Kyloh M, and Duffield M.** Identification of different types of spinal afferent nerve endings that encode noxious and innocuous stimuli in the large intestine using a novel anterograde tracing technique. *PloS one* 9: e112466, 2014.
42. **Storkholm JH, Villadsen GE, Jensen SL, and Gregersen H.** Mechanical properties and collagen content differ between isolated guinea pig duodenum,

jejunum, and distal ileum. *DigDisSci* 43: 2034-2041, 1998.

43. **Tanaka T, Shinoda M, Feng B, Albers KM, and Gebhart GF.** Modulation of visceral hypersensitivity by glial cell line-derived neurotrophic factor family receptor α -3 in colorectal afferents. *American journal of physiology Gastrointestinal and liver physiology* 300: G418-424, 2011.
44. **Yang J, Liao D, Zhao J, and Gregersen H.** Shear modulus of elasticity of the esophagus. *Annals of biomedical engineering* 32: 1223-1230, 2004.
45. **Yang J, Zhao J, Liao D, and Gregersen H.** Biomechanical properties of the layered oesophagus and its remodelling in experimental type-1 diabetes. *Journal of biomechanics* 39: 894-904, 2006.
46. **Yang J, Zhao J, Nakaguchi T, and Gregersen H.** Biomechanical changes in oxazolone-induced colitis in BALB/C mice. *Journal of biomechanics* 42: 811-817, 2009.
47. **Yu J, Zeng Y, Zhao J, Liao D, and Gregersen H.** Quantitative analysis of collagen fiber angle in the submucosa of small intestine. *Computers in biology and medicine* 34: 539-550, 2004.
48. **Zeng YJ, Qiao AK, Yu JD, Zhao JB, Liao DH, Xu XH, and Hans G.** Collagen fiber angle in the submucosa of small intestine and its application in Gastroenterology. *World journal of gastroenterology* 9: 804-807, 2003.
49. **Zhao JB, Sha H, Zhuang FY, and Gregersen H.** Morphological properties and residual strain along the small intestine in rats. *World journal of gastroenterology* 8: 312-317, 2002.
50. **Zhou Q and Verne GN.** New insights into visceral hypersensitivity--clinical implications in IBS. *NatRevGastroenterolHepatol* 8: 349-355, 2011.

Fig. 1

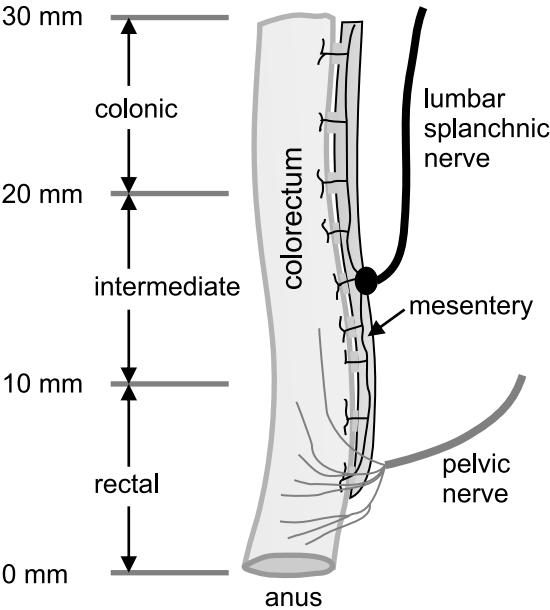


Fig. 2

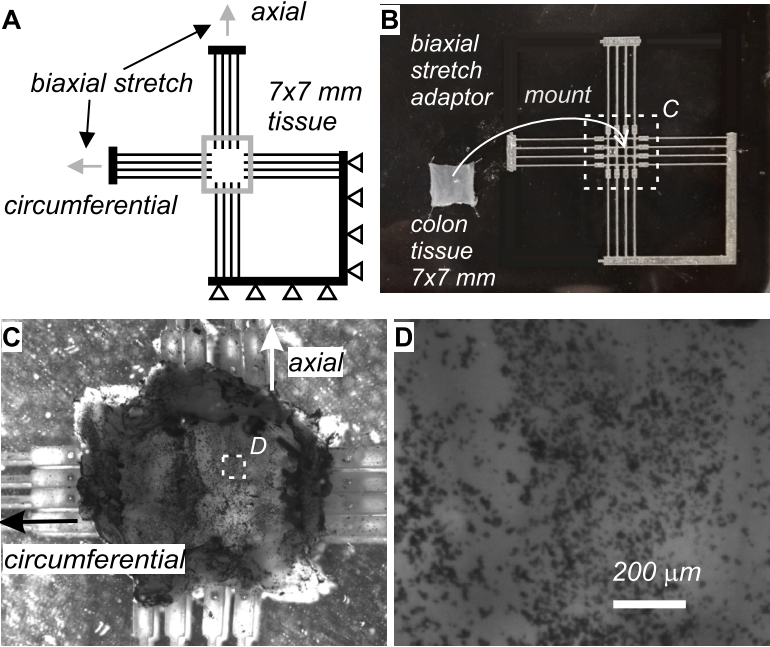


Fig. 3

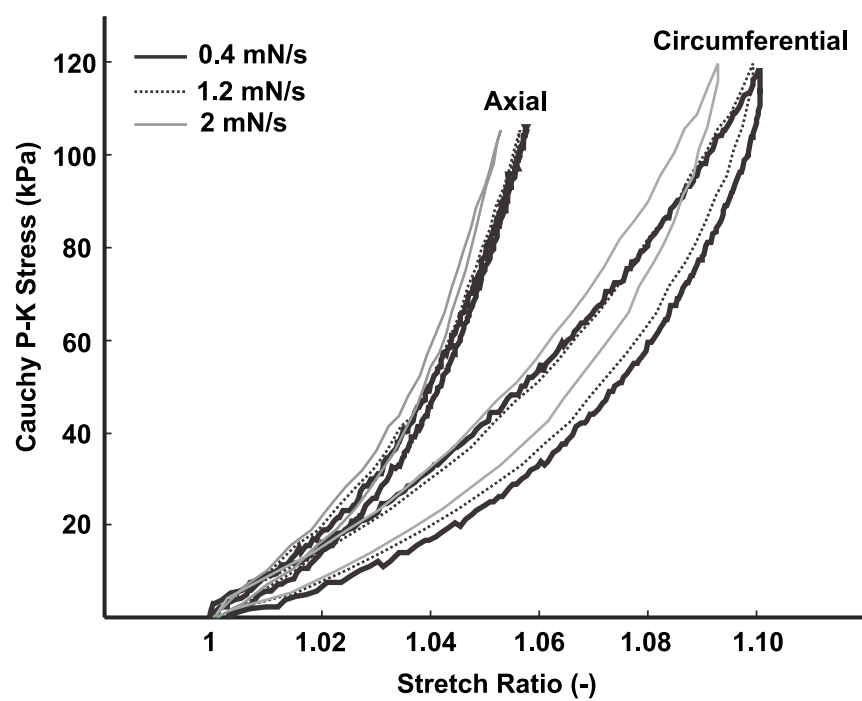


Fig. 4

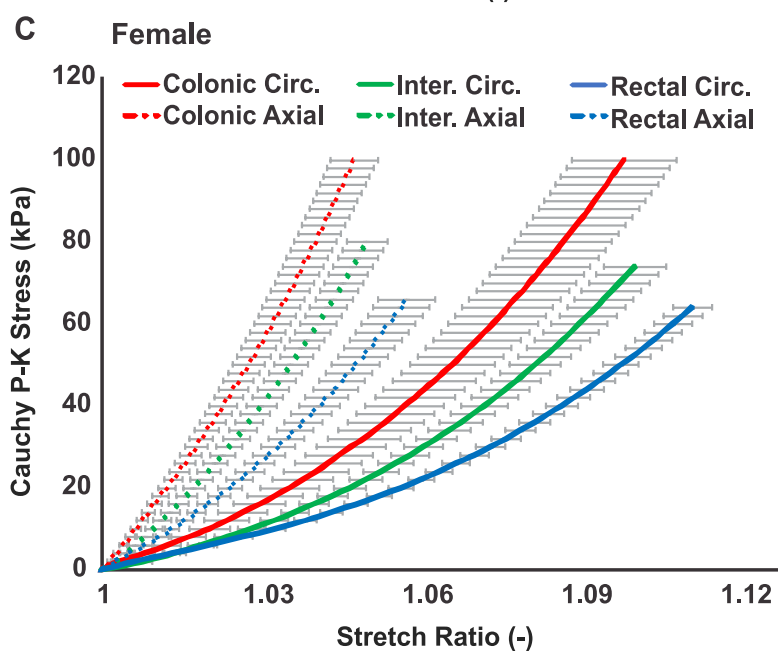
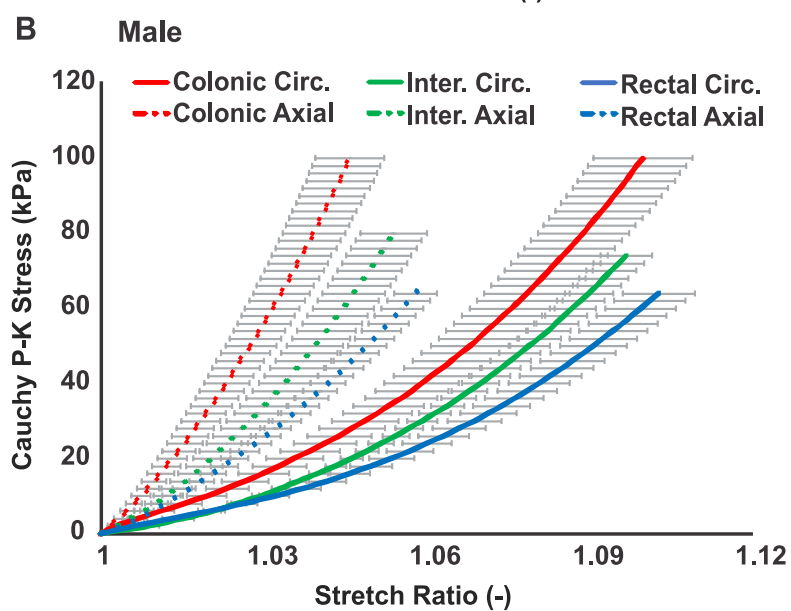
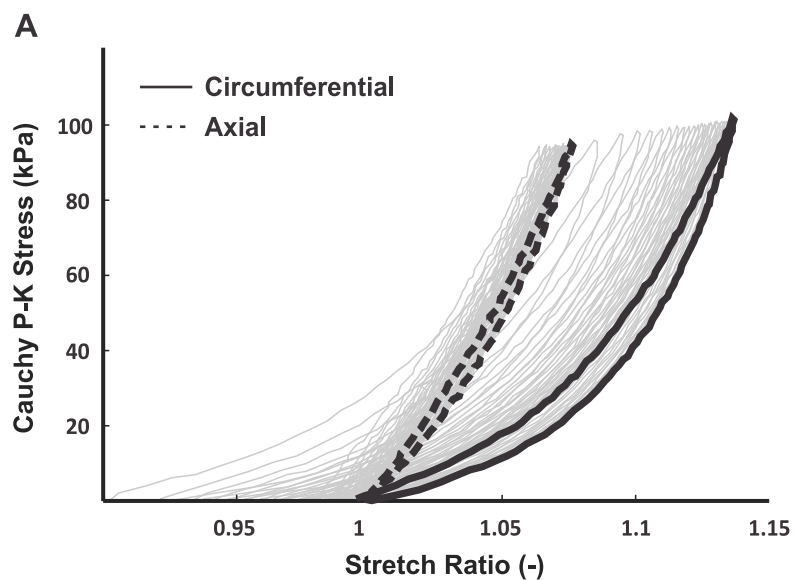


Fig. 5

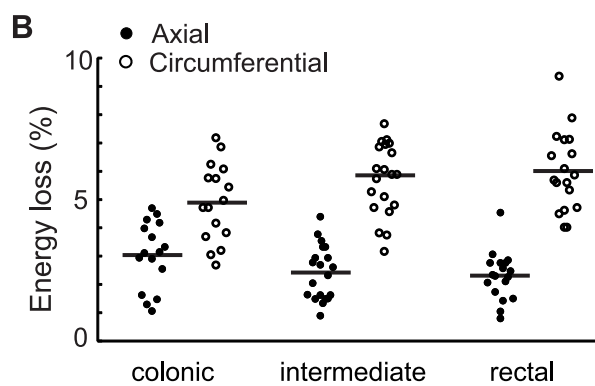
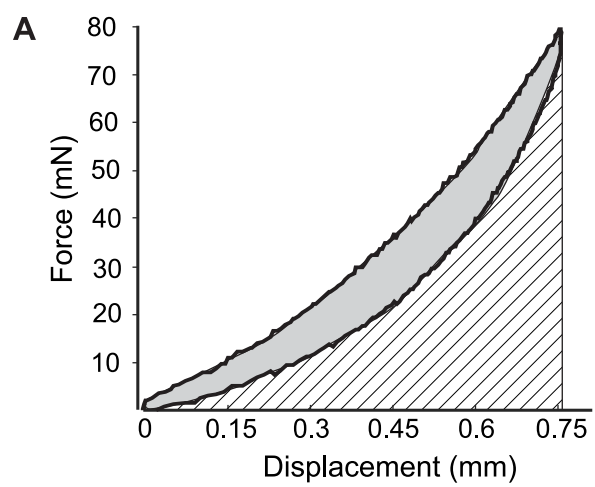
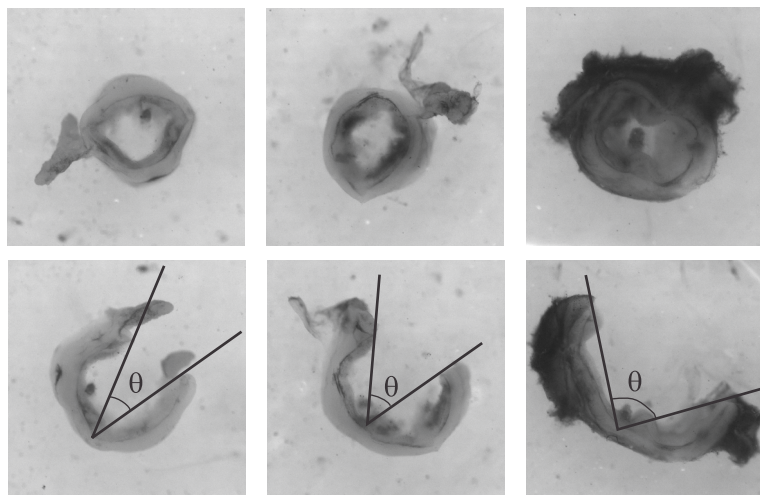


Fig. 6

A



B

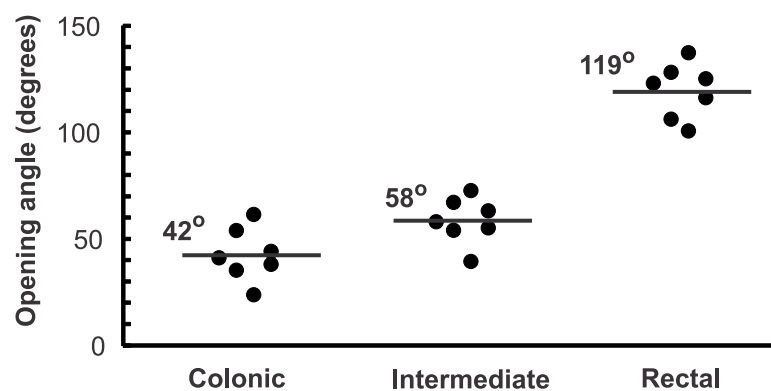


Fig. 7

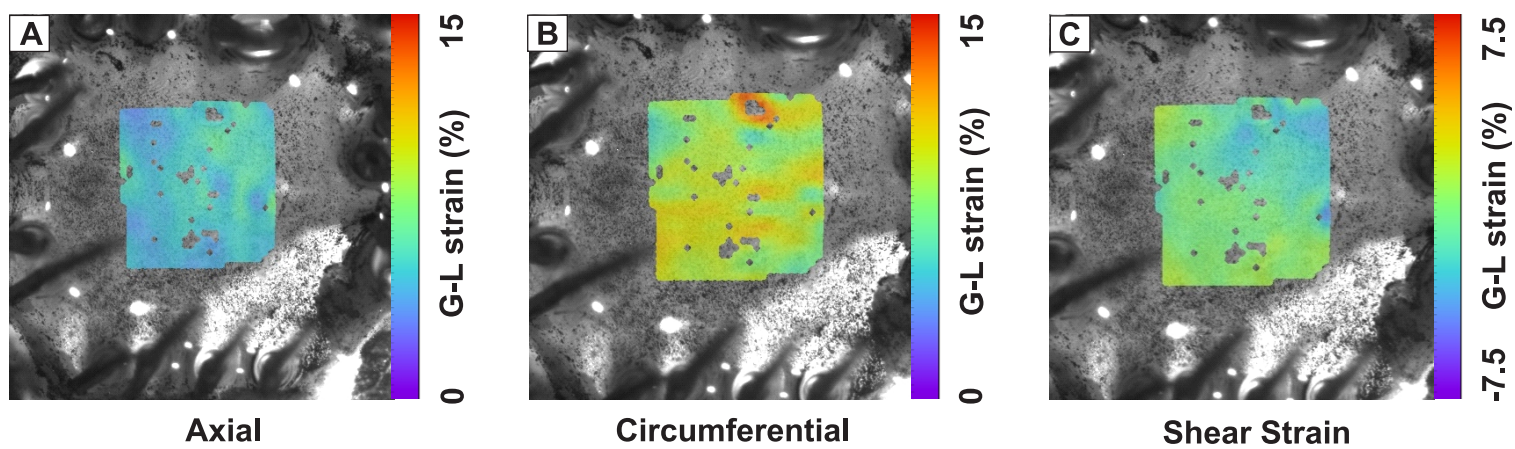


Fig. 8

