In vivo reference point indentation reveals positive effects of raloxifene on mechanical properties following six months of treatment in skeletally mature beagle dogs.

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ABSTRACT

Raloxifene treatment has been shown previously to positively affect bone mechanical properties following one year of treatment in skeletally mature dogs. Reference point indentation (RPI) can be used for *in vivo* assessment of mechanical properties and has been shown to produce values that are highly correlated with properties derived from traditional mechanical testing. The goal of this study was to use RPI to determine if raloxifene-induced alterations in mechanical properties occurred after 6 months of treatment. Twelve skeletally mature female beagle dogs were treated for 6 months with oral doses of saline vehicle (VEH, 1 ml/kg/day) or a clinically relevant dose of raloxifene (RAL, 0.5 mg/kg/day). At six months, all animals underwent *in vivo* RPI (10 N force, 10 cycles) of the anterior tibial midshaft. RPI data were analyzed using a custom MATLAB program, designed to provide cycle-by-cycle data from the RPI test and validated against the manufacturer-provided software. Indentation distance increase (IDI), a parameter that is inversely related to bone toughness, was significantly lower in RAL-treated animals compared to VEH (-16.5%) suggesting increased bone toughness. Energy absorption within the first cycle was significantly lower with RAL compared to VEH (-21%). These data build on previous work that has documented positive effects of raloxifene on material properties by showing that these changes exist after 6 months.

INTRODUCTION

The ultimate goal in treating patients with osteoporosis is to prevent fracture. How best one achieves this goal can be debated, but it is clear that increasing bone's mechanical properties is an essential component of any treatment regimen. Although we know mechanical properties are important, the challenge lies in their clinical assessment. Most often, bone mineral density (BMD) is used as a surrogate for fracture risk (and by extension bone mechanical properties) but the limitations of BMD on an individual patient basis are clear [1]. One example of this discordance between BMD and fracture risk is observed with raloxifene, which minimally affects BMD yet significantly reduces fracture risk [2,3].

Reference point indentation (RPI) has been recently introduced to the field as a tool for assessing mechanical properties of bone [4]. Pre-clinical studies have documented that a strong correlation exists between RPI outcomes, such as indentation distance increase (IDI) and mechanical property variables (modulus of toughness) estimated by three-point bending mechanical tests [5]. Although the device can be used on specimens *ex vivo*, the novel and exciting aspect is its potential application *in vivo*. Clinically, RPI-assessed IDI has been shown to distinguish between fracture and non-fracture patients [6] [7].

Previous work in our laboratory has documented that raloxifene, a selective estrogen receptor modulator, produces a positive effect on the intrinsic biomechanical properties of bone tissue, both cortical and cancellous, independently of bone mass after 1 year of treatment [8,9]. The goal of this study was to use RPI to test the hypothesis that raloxifene-induced improvements in material mechanical properties exist after 6 months of treatment.

METHODS

Experimental design

Twelve skeletally mature female beagles (1-2 years old) were separated into two groups (n = 6 per group) by matching body weights. Dogs were treated daily with either oral vehicle (saline, 1 mL/kg) or raloxifene (0.5 mg/kg). Raloxifene was dissolved in 10% hydroxypropyl-β-cyclodextrin and administered at a dose consistent with the clinical management of post-menopausal osteoporosis. This dose has been shown previously to alter mechanical properties in this animal model following one year of treatment [8,9]. After six months of treatment, all animals underwent *in vivo* mechanical property testing using RPI. As this study is part of a larger experiment, these same animals are continuing treatment and will be sacrificed after 12 months of treatment. All procedures were approved by the Indiana University School of Medicine Animal Care and Use Committee prior to the start of the study.

Reference point indentation (RPI)

Material-level mechanical properties of the anterior surface in the mid-diaphysis of the tibia cortex were assessed *in vivo* using RPI (Biodent Hfc, Active Life Scientific, Santa Barbara, CA). This site was chosen as it has been utilized previously in human *in vivo* testing [6] [7] and its limited soft tissue coverage facilitates easy access to the bone surface. The remodeling rate at this bone site is not known, yet the distal tibia at this age has an intracortical remodeling rate of ~1-2%/year [10] and <5% of the periosteal surface actively forming bone. Dogs were placed under general anesthesia using intravenous propofol and the skin over the right anterior tibia was shaved and aseptically prepared. The tibia mid-diaphysis was identified as the linear midpoint between the superomedial margin of the medial tibia condyle and the distomedial margin of the medial malleolus. A local anesthetic was injected just beneath the skin in the region of testing, just proximal to the midpoint of the tibia. Skin overlying the region was pierced with a sterile BP1 probe contained within the measurement head unit (MHU) attached to a modified holder apparatus (**supplementary Figure 1**). The MHU was lowered vertically, normal to the surface of the bone, until the probe assembly rested on the bone surface

(supplementary **Figure 2**). The periosteum was scraped from the underlying cortex by moving the reference probe across the bone surface. After removal of the periosteum, the reference probe was positioned, a reference force of ~13 Newtons was applied to stabilize the MHU, and the measurement protocol was initiated. Measurements began with a series of four preconditioning cycles at a force of 1 N and a frequency of 5 Hz, and concluded with a series of 10 testing cycles at 10 N and 2 Hz. Up to five measurements, within a few mm of each other, were collected on each animal. If a test was found to be unusable during the live animal testing, a replacement was run. In cases where the data was found after the fact to be implausible (for instance a negative IDI that was not caught during the in vivo test) it was not used in the analysis leaving some animals with less than five tests. The coefficient of variation within each animal is presented in supplementary Table 1. The animals were conscious and mobile within 30 minutes post-testing. There was no sign of pain or discomfort based on pain scoring taken within the first 8-12 hours post test, and then again 24 hours post test.

MATLAB code

Raw data output from the RPI analysis software (version 2.0) were imported into a customized MATLAB code (Mathworks). The code was internally developed to supplement the RPI software by providing cycle-by-cycle data, which is not available in the manufacturer-supplied software. For example, the manufacturer software provides averages for the unloading slope and energy parameters between cycles 3 and 10. We were interested in the actual values for these parameters (not the averages) and also what the values looked like in the first cycles. To develop the code, both force versus time and distance versus time data were used to produce the points associated with each cycle's curve, from which primary parameters were determined (**see supplementary figure 3**). The code was validated by comparing its output to the standard RPI analysis software for first cycle indentation distance (ID), total indentation distance (TID), indentation distance increase (IDI), first cycle unloading slope (US), average unloading slope

(cycles 1-10), and average energy dissipated (cycles 3-10). Upon validation, the MATLAB program was used to analyze RPI outcomes between the two treatment groups. Primary variables of interest from the MATLAB program are outlined in **Figure 1 and Table 1**.

Statistics

RPI data were evaluated using one-tailed independent samples *t-tests* because prior experiments consistently showed improvement in toughness in raloxifene-treated bone at different sites. One-tailed tests are therefore justified, and provide greater statistical power to detect differences between treatment groups, especially in cases of small sample sizes. Data obtained from the Biodent internal software were also compared to data obtained from the custom MATLAB code using the Pearson's product-moment correlation algorithm. For all statistical tests, *a priori* α-levels were set at 0.05.

RESULTS

The custom MATLAB code was validated against the manufacturer supplied software. First cycle ID, total ID, indentation distance increase (IDI), average energy (cycle 3-10) and first cycle unloading slope all had significant correlation coefficients of > 0.988 (**Figure 2 and supplementary Figure 4**). Average unloading slope (cycle 1-10) had a somewhat lower, yet still statistically significant correlation coefficient (0.964) (Supplementary Fig 4).

After six months of raloxifene treatment, IDI (-16.5%) first cycle ID (-30%), and TID (-29%) were all significantly lower than in vehicle-treated animals (p=0.008, 0.048, and 0.046, respectively) **(Figure 3 and Table 2)**. First cycle energy was significantly lower (-21%) with raloxifene treatment, whereas there was no difference between treatment groups in total energy (**Figure 3)**. There was no significant difference in first cycle unloading slope (p = 0.411) or creep indentation distance (p=0.149).

DISCUSSION

Clinical practice relies heavily on assessing bone mineral density to determine an individual's risk of fracture and their response to treatment. Although the utility of BMD for predicting fracture risk and determining response to treatment is valuable when applied to populations, limitations exist for individual patients [1]. These limitations have hindered progress toward individual patient fracture risk assessment. Techniques such as finite element modeling of high-resolution CT images show promise as a tool for patient-specific assessment of bone strength, rigidity and Young's modulus, and can be used to specifically model sites of high clinical relevance such as the femoral neck and vertebra, yet these techniques still only estimate mechanical properties [11]. The development of reference point indentation (RPI), a tool that directly measures bone material-level biomechanical properties, has the potential to supplement current clinical assessment by allowing direct measurements of biomechanical properties assuming that properties at the tibia have some relation to clinically-relevant sites. The ability to differentiate fracture versus non-fracture patients using RPI has been demonstrated [6] [7], and parameters such as indentation distance increase (IDI) highly correlate with mechanical properties from traditional laboratory tests [5]. The current study extends these findings by showing that RPI can detect *in vivo* alterations in bone material properties with drug treatment.

RPI integrates the material-level, or intrinsic, biomechanical properties of bone. The mechanical properties of a whole bone, often referred to as structural, or extrinsic, biomechanical properties, are determined by a combination of bone mass (how much bone there is) and bone quality, a composite term that encompasses several variables [12]. RPI parameters such as ID, TID, and IDI are thought to reflect the ability of the bone to resist the initiation and propagation of damage. IDI is inversely related to crack growth toughness measured by R-curve testing [6] and modulus of toughness measured by 3-point bending [5]. Larger IDI values indicate that a bone is less able to resist damage, as the probe penetrates

further into the matrix with repeated loading. Results here show that raloxifene treatment produced a lower IDI, effectively toughening the bone by improving the material-level ability of the bone to resist production and propagation of damage. This finding is in line with previous data from our laboratory showing enhanced modulus of toughness with raloxifene at the vertebra, femoral neck, and femoral diaphysis after 1 year of treatment [8,9]. Importantly, the current work shows that changes in material-level properties can be detected as early as 6 months and can be measured with the minimally invasive RPI device. On-going work in our laboratory suggests these positive effects on mechanical properties are due to raloxifene-induced increases in skeletal hydration.

In addition to measures of indentation depth, the cyclic nature of the RPI test allows assessment of energy, represented by the area under the force-displacement curve. Manufacturer-supplied RPI software outputs energy data as the average energy dissipation of cycles 3-10. As the majority of damage is incurred during the first cycle of the test ($ID^{(1st)}$ on average is 96% of TID), we wanted to examine energy parameters of the first and each of the subsequent individual cycles. Using a custom built MATLAB code, written to deconstruct the RPI test down to cycle-by-cycle data, we found that energy absorption was significantly smaller in raloxifene-treated animals during cycle one and that there were no significant differences between groups for any of the remaining nine cycles. This is consistent with the significantly smaller first cycle ID, as a lower indentation depth would be expected to produce less energy to be absorbed. These differences in first cycle energy absorption drove the trend toward differences in total energy (the sum of energy over the course of the 10 cycles). These data indicate that cycle-by-cycle energy analyses provide a useful supplement to the data provided by standard manufacturer software.

Our results should be interpreted in the context of a few limitations. While the value of RPI for detecting treatment-induced differences has been shown, this is shown here only for

raloxifene and may not hold true for other treatments. Our small samples size resulted in some parameters failing to differ statistically. However, despite having only six animals per group, several parameters such as IDI, first cycle energy, first cycle ID, TID, did show significant differences between treatment groups. Post hoc power analyses reveal those parameters that did not reach statistical significance between groups all had power less than 0.20, while IDI had a power of 0.788. We do not have an assessment of periosteal formation in these animals and thus cannot discount that the mean tissue age at the site was different. We have examined tibial sections from age-matched dogs and have shown that < 5% of the periosteal surface is actively forming bone, but it is not known whether raloxifene alters this activity. Finally, given that this was an interim *in* vivo investigation, we do not have other data that would complement the analysis such as how raloxifene affects remodeling, density, or traditional mechanical properties from monotonic testing.

In conclusion we have shown that raloxifene-induced improvements in mechanical properties exist after 6 months of treatment in skeletally mature dogs. Further, these results highlight the value of RPI as an analytical tool for measuring biomechanical properties of bone *in vivo*.

Acknowledgements.

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Table 1. Reference point indentation (RPI) parameters from custom MATLAB code

Variable	**Abbreviation**	**Description**
1st cycle Indentation distance	$ID^{(1st)}$	Probe penetration depth on first cycle
1st cycle Energy	$Energy^{(1st)}$	Energy dissipated during first cycle
1st cycle unloading slope	$US^{(1st)}$	Slope of unloading portion of first cycle
1st cycle creep indentation distance	$CID^{(1st)}$	Distance during hold portion of first cycle
Indentation distance increase	IDI	Relative difference between 1st and 10th cycle ID.
Total indentation distance	TID	Probe penetration depth after 10th cycle
Total energy	$Energy^{(Total)}$	Total energy dissipated over 10 cycles

Table 2. *In vivo* Reference Point Indentation values of the tibia diaphysis following 6-months of treatment. Data shown as means and standard deviations.

Variable	**Vehicle (n=6)**	**Raloxifene (n=6)**	**P value**
$ID^{(1st)}$, μm	166 ± 61	116 ± 24	0.048
$Energy^{(1st)}$, μJ	449 ± 94	354 ± 60	0.032
$US^{(1st)}$, N/μm	0.457 ± 0.025	0.446 ± 0.035	0.271
$CID^{(1st)}$, μm	8.37 ± 0.75	7.67 ± 1.37	0.149
IDI, μm	13.9 ± 1.4	11.6 ± 1.2	0.008
TID, μm	173 ± 62	122 ± 24	0.046
$Energy^{(Total)}$, μJ	944 ± 150	852 ± 155	0.161

ID(1st), first cycle indentation distance; Energy(1st), first cycle energy; US(1st), first cycle unloading slope; CID(1st), first cycle creep indentation distance; IDI, indentation distance increase; TID, total indentation distance; Energy(Total), total energy.

Figure Legends

Figure 1. RPI output from custom MATLAB code used to analyze cycle-by-cycle data. Cycles one and ten are highlighted for reference yet all cycles were included in the analysis.

Figure 2. Correlation plots for RPI manufacturer software and custom MATLAB code. First cycle ID (A) show the strongest relationships between analysis tools. IDI (B) had a marginally lower R value, likely due to rounding of values in the RPI analysis software. More notable scatter exists for average energy (C). Upon further examination it was found that the RPI software was overestimating energy in some tests (n=8) due to the system incorrectly identifying the final part of cycle 10. When these eight tests were removed from the analysis, the R value was 0.998.

Figure 3. Raloxifene positively affects bone material properties of the anterior tibial mid-shaft cortex after six months of treatment. *In vivo* assessment of indentation distance increase (IDI) was significant lower in RAL-treated animals compared to VEH (A). First cycle ID (B), total ID (C), and first cycle energy (D) were also all significantly lower in RAL compared to VEH. Box and wisker plots represent the interquartile range (the box) and 1.5x this range (wiskers). N= 6 animals per treatment group.

Supplementary Figures

Supplementary Figure 1. The experimental setup of the BioDent Hfc was modified to facilitate *in vivo* assessment of dogs. The measurement stand supplied with the equipment, which holds steady the measurement head unit (MHU) in the X and Y coordinates, was replaced with an articulated boom from a dissection microscope. This setup facilitated steady lateral movements (X and/or Y coordinates) at a constant Z coordinate while scraping the periosteum from the test site. To accurately achieve the desired reference force prior to testing, a digital scale was placed directly underneath the articulated boom and MHU, and the dog's leg was secured in a custom fitted foam support between the digital scale and the MHU.

Supplementary Figure 2. Test set-up for *in vivo* RPI testing of dog tibia.

Supplementary Figure 3. Step-by-step flow chart explaining the MATLAB code.

Supplementary Figure 4. Additional coefficients of determination for RPI manufacturer software and custom MATLAB code. Total ID (A) and first cycle US (B) show the strongest relationships between analysis tools. More notable scatter exists for average unloading slope (C). Upon further examination it was found that the RPI software was underestimating slopes in some tests (n=8) due to the system incorrectly identifying the final part of cycle 10. When these eight tests were removed from the analysis, the R value was 0.982.

Supplementary Table 1.

Variable	*VEHICLE* *CV, %* *(min-max)*	*RALOXIFENE* *CV, %* *(min-max)*
$ID^{(1st)}$, µm	27 (16 – 44)	14 (2 – 32)
$Energy^{(1st)}$, µJ	33 (28 – 35)	16 (6 – 45)
$US^{(1st)}$, N/µm	5 (3 – 13)	5 (3 – 9)
$CID^{(1st)}$, µm	30 (11 – 46)	35 (11 – 72)
IDI, µm	19 (10 – 33)	19 (3 – 40)
TID, µm	26 (15 – 43)	13 (3 – 30)
$Energy^{(Total)}$, µJ	27 (21 – 36)	15 (6 – 34)

ID(1st), first cycle indentation distance; Energy(1st), first cycle energy; US(1st), first cycle unloading slope; CID(1st), first cycle creep indentation distance; IDI, indentation distance increase; TID, total indentation distance; Energy(Total), total energy.

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Figure 1

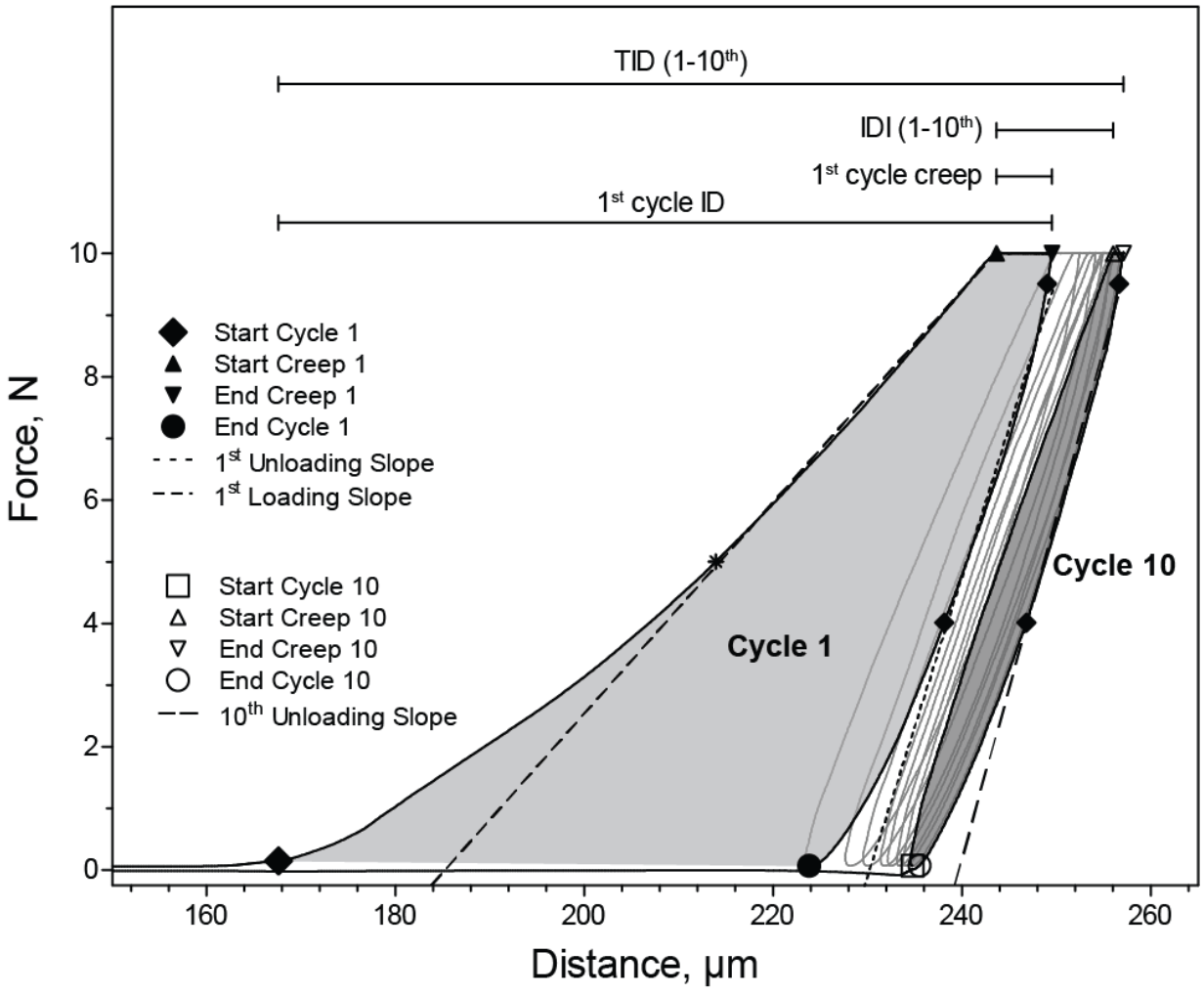
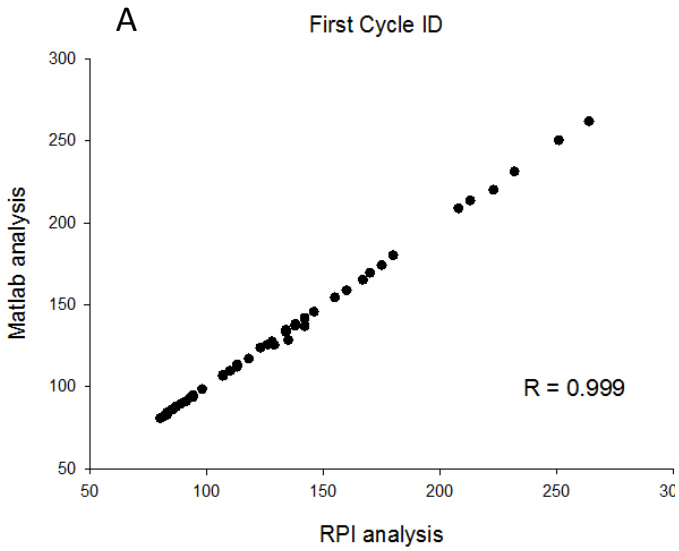
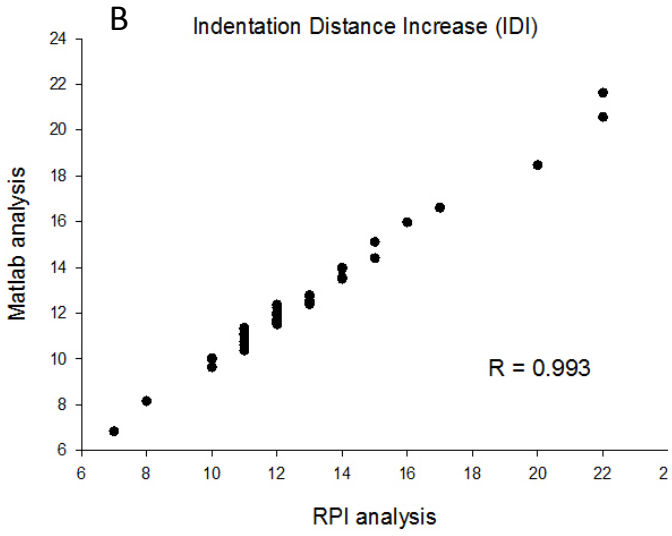

TID (1-10th)
IDI (1-10th)
1st cycle creep
1st cycle ID
Start Cycle 1
Start Creep 1
End Creep 1
End Cycle 1
1st Unloading Slope
1st Loading Slope
Start Cycle 10
Start Creep 10
End Creep 10
End Cycle 10
10th Unloading Slope
Cycle 1
Cycle 10
Force, N
Distance, µm
0
2
4
6
8
10
160
180
200
220
240
260

Figure 2


A
First Cycle ID
Matlab analysis
RPI analysis
R = 0.999
300
250
200
150
100
50
50
100
150
200
250


B
Indentation Distance Increase (IDI)
Matlab analysis
RPI analysis
R = 0.993
24
22
20
18
16
14
12
10
8
6
6
8
10
12
14
16
18
20
22

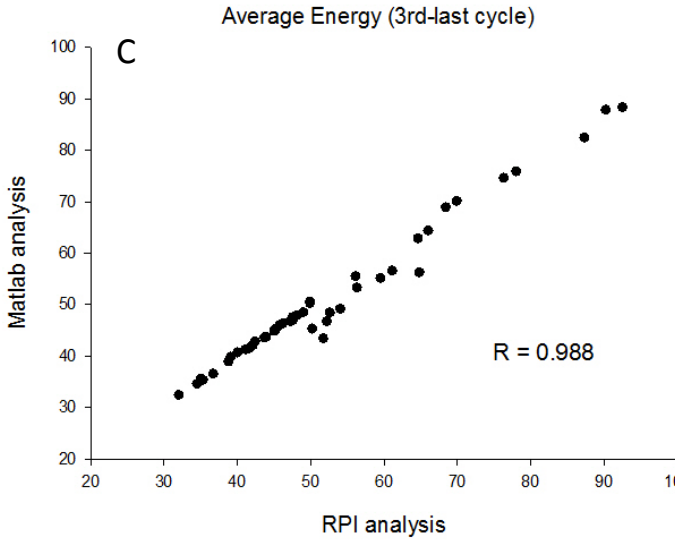
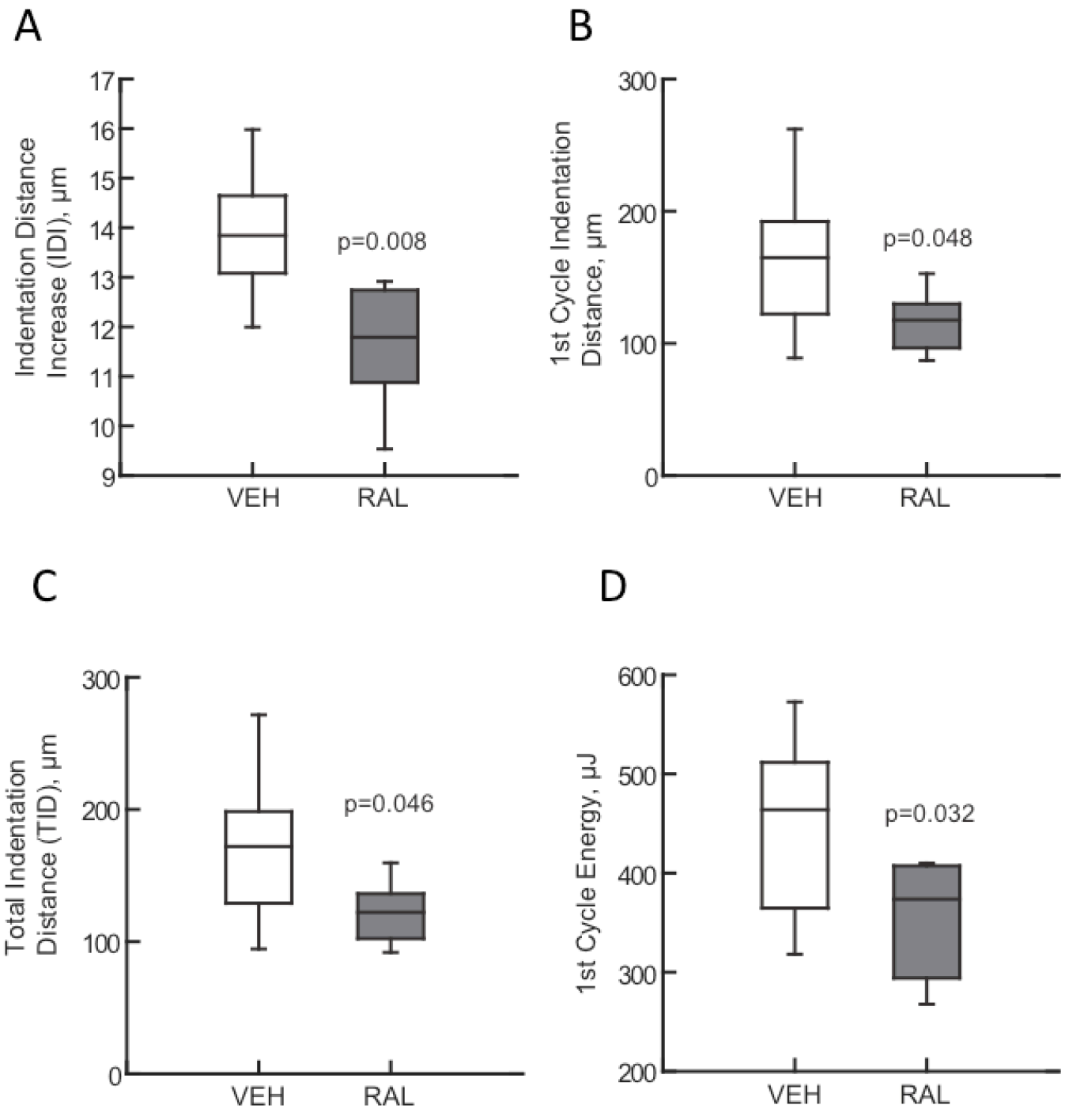
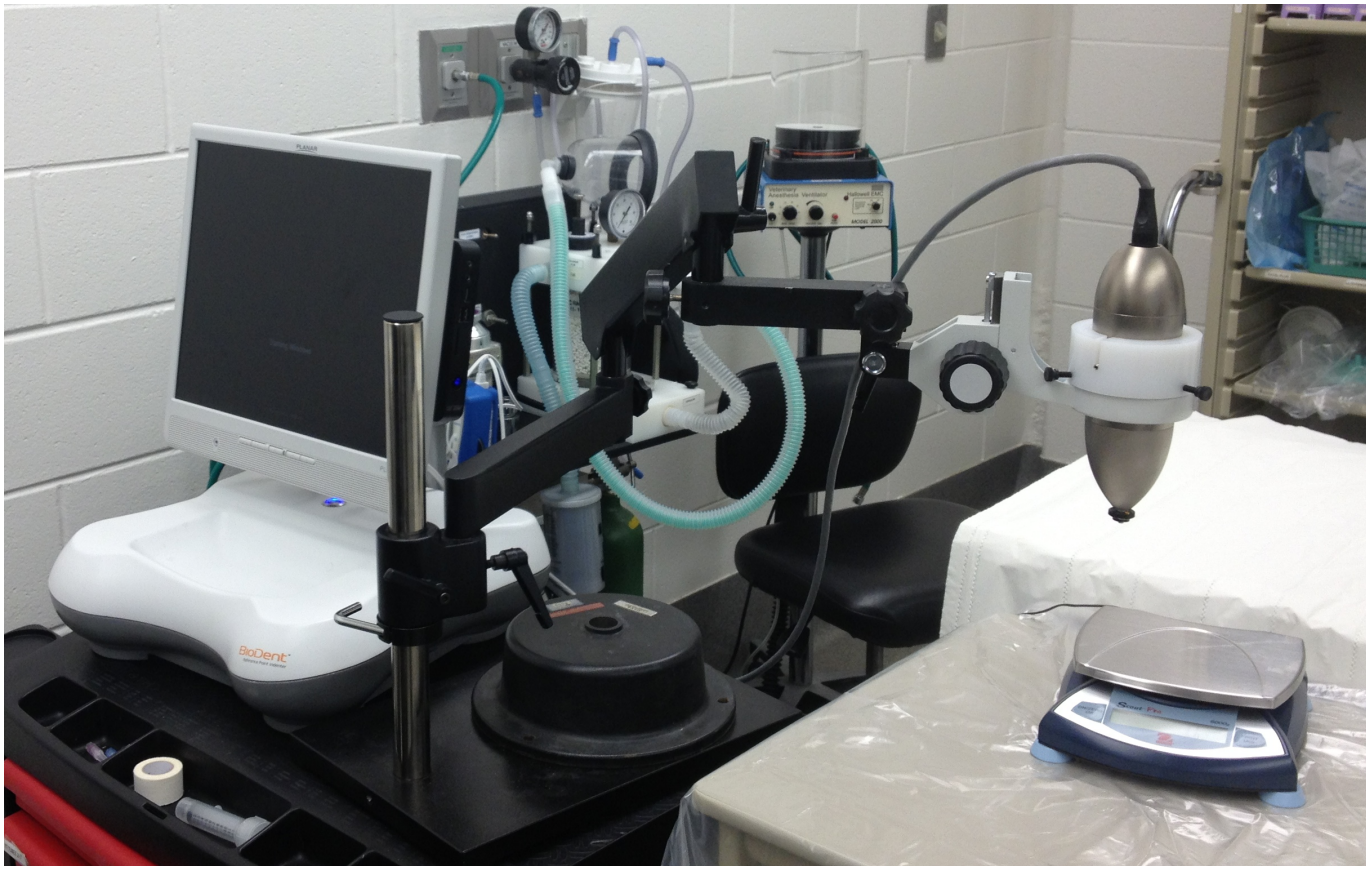

Average Energy (3rd-last cycle)
C
Matlab analysis
RPI analysis
R = 0.988
100
90
80
70
60
50
40
30
20
20
30
40
50
60
70
80
90

Figure 3

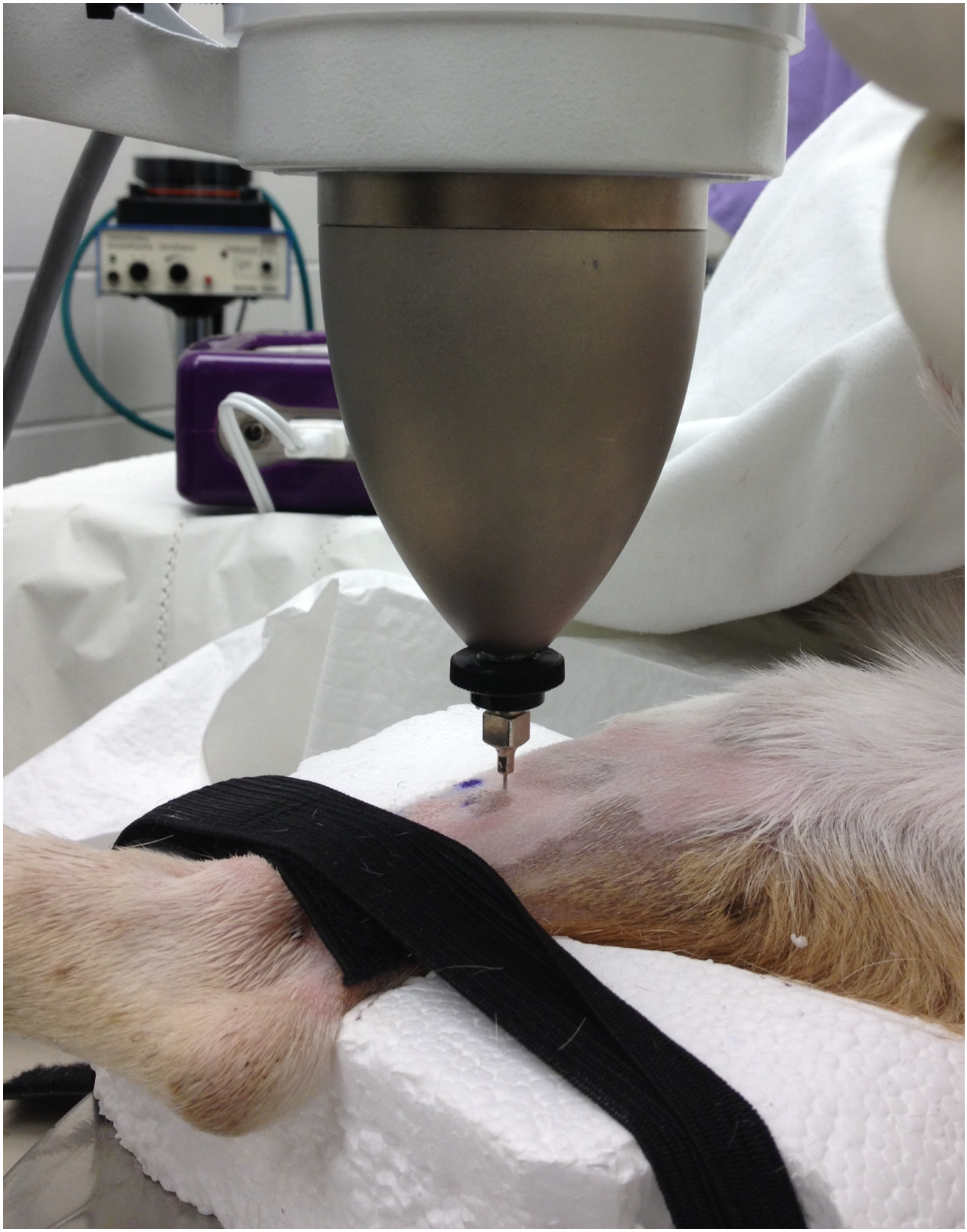

A
Indentation Distance Increase (IDI), µm
17
16
15
14
13
12
11
10
9
p=0.008
VEH
RAL
B
1st Cycle Indentation Distance, µm
300
200
100
0
p=0.048
VEH
RAL
C
Total Indentation Distance (TID), µm
300
200
100
0
p=0.046
VEH
RAL
D
1st Cycle Energy, µJ
600
500
400
300
200
p=0.032
VEH
RAL

Supplementary Figure 1



The experimental setup of the BioDent Hfc was modified to facilitate *in vivo* assessment of large animals. The measurement stand supplied with the equipment, which holds steady the MHU in the X and Y coordinates, was replaced with an articulated boom from a dissection microscope. This setup facilitated steady lateral movements (X and/or Y coordinates) at a constant Z coordinate while scraping the periosteum from the test site. To accurately achieve the desired reference force prior to testing, a digital scale was placed directly underneath the articulated boom and MHU, and the dog's leg was secured in a custom fitted foam support between the digital scale and the MHU.

Supplementary Figure 2



Suppl Figure 3

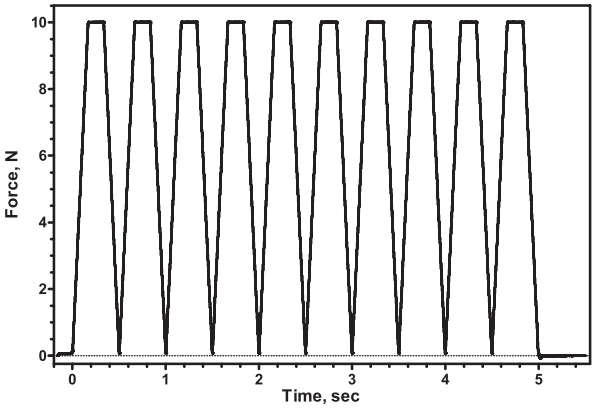
STEP BY STEP PROCESS OF THE MATLAB CODE

CURVES ARE DEFINED USING FORCE-TIME DATA

-The offset is fixed by adding the absolute value of the first point to the force array

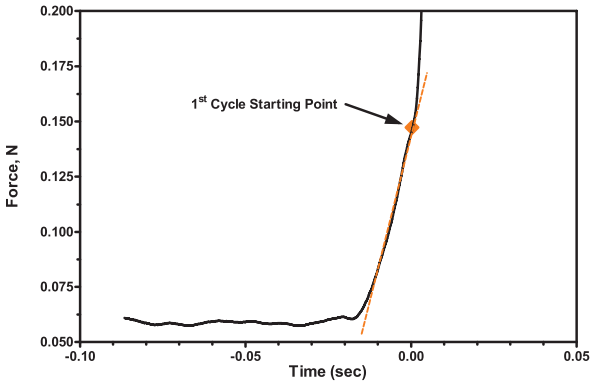
-The force and distance data is smoothed using Savitzky-Golay filtering method with polynomial order 3 and frame size of 51

-The derivative of each point in the force array is calculated to determine the slope



TOUCHDOWN POINT DETERMINATION

-The 1st cycle starting point is defined as the last point in the first 7% of the force derivative that has a value less than 0.002

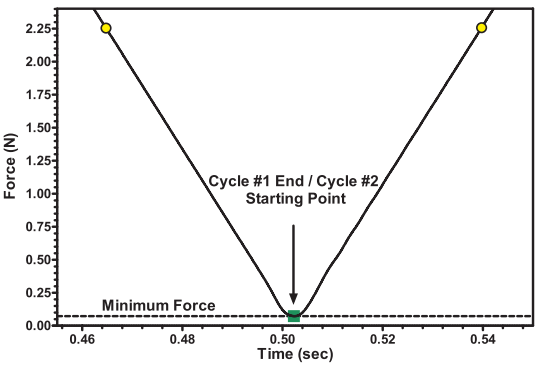


DEFINING EACH CYCLE

-The end of each cycle / beginning of the next cycle (local minima) is defined as the point that is less than the 150 points before it and the 150 points after it.

-The final point (end of last cycle) is the last point where the force is greater than the average of the forces at the local minima

-The dataset is then divided into cycles

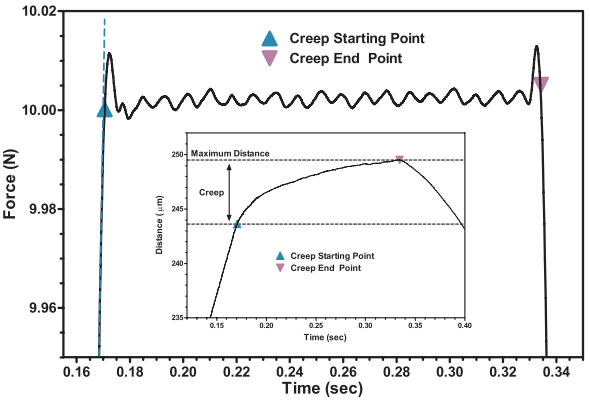


DEFINING CREEP INDENTATION DISTANCE

-The first point of the creep region is defined as the first portion of the force that has a derivative less than 0.001

-The last point in the creep region is defined as the point where the the distance is at a maximum (determined using the Distance-Time graph).

-This is repeated for each cycle



Suppl Figure 3 cont.

STEP BY STEP PROCESS OF THE MATLAB CODE (Cont.)

CALCULATION OF CYCLE-BY-CYCLE DATA

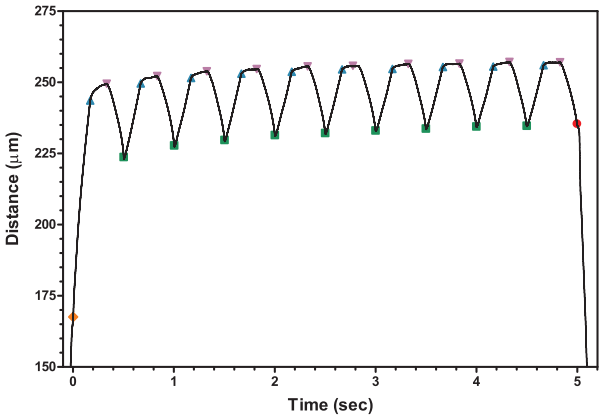
-This is done using both Distance vs. Time and Force vs. Distance plots.

DISTANCE versus TIME

This graph is used to calculate:

- CID: Creep Indentation Distance
- ID: Indentation Distance
- IDI: Indentation Distance Increase

-TID: Total Indentation Distance

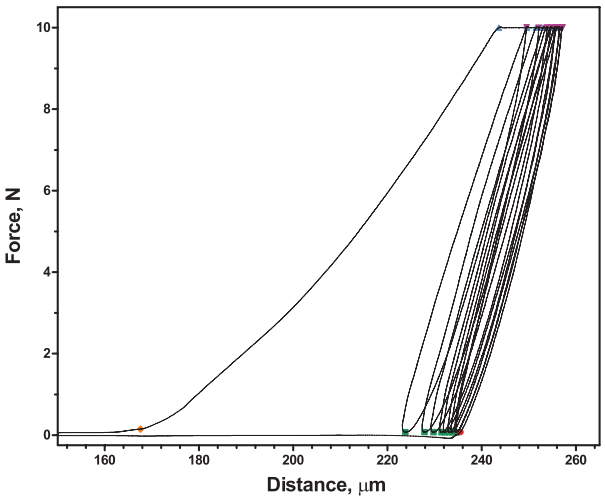


FORCE versus DISTANCE

This graph is used to calculate

-Energy: Area under the curve

-US: Unloading Slope



Supplementary Figure 4