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Establishing a Robust Ligature-Induced Model of Murine Periodontitis for Evaluation of Oral Neutrophils

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Dr. Jaydev Upponi
Science Editor
Journal of Visualized Experiments
1 Alewife Center, Suite 200
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January 7, 2019

Dear Dr. Upponi,

Re: Submission of manuscript titled, **"**Establishing a Robust Ligature-Induced Model of Murine Periodontitis for Evaluation of Oral Neutrophils**"**

Please find enclosed our manuscript, which we would like to submit to the Journal of Visualized Experiments.

The authors (listed below) have compiled a manuscript that presents an alternative ligature-induced model of murine periodontitis which highlights the rationale for several modifications of existing protocols to increase volumes of inflamed tissue and decrease animal usage. A novel technique to assess oral neutrophils in an analogous manner to human subjects is also explored.

1. Jeffrey Chadwick: PhD Candidate, University of Toronto
2. Michael Glogauer: Professor, University of Toronto (Corresponding Author)

We certify that work herein is original, not under publication consideration elsewhere, and the authors are free of any financial conflicts of interest. Both authors have approved the manuscript and agree with its submission to the Journal of Visualized Experiments.

We appreciate the opportunity for the manuscript to be considered and we look forward to hearing from you at your earliest convenience.

Yours truly,

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TITLE:

Robust Ligature-Induced Model of Murine Periodontitis for the Evaluation of Oral Neutrophils

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KEYWORDS:

periodontitis, ligature, gingiva, periodontium, alveolar bone, animal model, flow cytometry, neutrophil, immunology, inflammation

SUMMARY:

This article presents a protocol for establishing a ligature-induced model of murine periodontitis involving multiple maxillary molars, resulting in larger areas of the involved gingival tissue and bone for subsequent analysis as well as reduced animal usage. A technique to assess oral neutrophils in a manner analogous to human subjects is also described.

ABSTRACT:

The main advantages of studying the pathophysiology of periodontal disease utilizing murine models are highlighted by the reduced cost of animals, array of genetically modified strains, the vast number of analyses that can be performed on harvested soft and hard tissues. However, many of these systems are subject to procedural criticisms. As an alternative, the ligature-induced model of periodontal disease, driven by the localized development and retention of a dysbiotic oral microbiome, can be employed, which is rapidly induced and relatively reliable. Unfortunately, the variants of ligature-induced murine periodontitis protocol are isolated to focal regions of the periodontium and subject to premature avulsion of the installed ligature. This minimizes the amount of tissue available for subsequent analyses and increases the number of animals required for the study. This protocol describes the precise manipulations required to place extended molar ligatures with improved retention and usage of a novel rinse protocol to recover oral neutrophils in mice with an alternative approach that mitigates the aforementioned technical challenges.

INTRODUCTION:

Periodontal disease (PD) is an osteolytic condition associated with significant host morbidity

and economic burden, which is manifested by gingival inflammation and loss of both soft tissue attachment and osseous support for the affected dentition[1–4]. This process is governed by interactions between the oral microbiota and innate immune system of the host. It is also associated with exacerbation of other systemic inflammatory diseases including diabetes, cardiovascular disease, and cancer[5–8]. Historically, it was hypothesized that PD pathogenesis is dependent on large quantities of specific bacteria such as *Porphyromonas gingivalis*[9]. However, recent evidence suggests that the microbial component of PD is mediated by the dental biofilm. The biofilm is an organized, complex community of numerous microorganisms that can exist in healthy symbiotic and destructive dysbiotic states[10,11]. The oral biofilm normally affords resistance to the host by preventing the establishment of foci of pathogenic bacteria and promotes ideal gingival tissue structure and function through regulation of the host immune response[12,13]. Perturbations of the equilibrious relationship between commensal organisms within the oral cavity and the host immune system may lead to alterations in tissue homeostasis, resulting in dysbacteriosis and development of the hallmark clinical and radiographic appearances of PD[5,10,12–14].

Interestingly, the establishment of an oral dysbacteriosis, while required for the initiation of PD, is not sufficient to drive PD in all individuals, eluding toward the ability of the host immune response to subvert the transition of microbiota between symbiotic and dysbiotic states[15]. This places a particular spotlight on the means through which PD influences one of the leading characters of the innate immune system [i.e., the polymorphonuclear granulocyte (PMN), or neutrophil] from local and systemic perspectives[16,17].

In humans, PMNs are recruited from the circulation at a rate of ~2 x 10^6 cells/h in healthy periodontal connective tissues, where they are the predominating leukocyte population. Here, they are subsequently expelled from the gingival sulcus into the oral cavity in the form of gingival crevicular fluid. In the presence of PD, neutrophilia manifests within the circulation and oral cavity, where these effector cells possess a hyperinflammatory phenotype that leads to the aforementioned destruction of the periodontium[17–22]. Therefore, understanding the role of PMNs in PD and other systemic inflammatory conditions is of utmost importance.

Although it is widely accepted that chronic diseases are reciprocally linked to PD, the underlying mechanisms have yet to be elucidated, contributing to difficulties in the management of these morbid and potentially fatal systemic conditions. Multiple experimental animal models, each with unique advantages and disadvantages, have been utilized to study the pathophysiology of PD[23,24]. Focusing specifically on murine models, there are a variety of protocols through which the study of PD is facilitated; however, they possess several technical and physiologic shortcomings[25–31].

First, the oral gavage mouse model requires numerous oral inoculations of human periodontal pathogens to generate gingival inflammation and bone loss. Additionally, it is generally preceded by a period of antibiotic treatment to subvert the murine commensal oral flora[25]. This model often requires specialized training to safely perform the oral gavage, uses only a small

fraction of periodontal pathogens from the more complex human oral microbiome, and requires several months to establish alveolar bone loss.

In contrast, chemically induced murine models utilize the oral delivery of trinitrobenzene sulfonic acid (TNBS) or dextran sulfate sodium (DSS), agents commonly used in establishing murine models of colitis over a period of several months to induce periodontal bone loss[26]. Intraoral and extraoral abscess-based models are available, which involve the murine incisors and tissues of the dorsum as well as calvarium, respectively. In the former abscess model, several injections of bacteria are administered, creating multiple gingival abscesses and a dearth of alveolar bone loss, limiting their use in the study of PD. The latter abscess models are significantly more apt to studying bacterial virulence, inflammation, and bone resorption at sites outside of the oral cavity, which eliminates evaluation of the periodontium and oral microbiome[27–31].

Using the ligature-induced model of periodontitis, a braided silk suture has commonly been installed circumferentially around the second molar. As an alternative, a single linear segment of suture material can be inserted between the first and second molars[32,33]. The goal of the ligature placement is to facilitate bacterial accumulation and generate dysbiosis within the gingival sulci, resulting in periodontal tissue inflammation and destruction of the tissues composing the periodontium. Most notably, this model is capable of producing significantly more alveolar bone loss compared to the more commonly used oral gavage model[34]. Further complicating the use of the oral gavage model is the natural resistance by several strains of mice (i.e., C57BL/6) to developing alveolar bone loss. This is also problematic, as this strain is the most frequently used in murine-based animal research[35].

Existing procedures described by Marchesan et al. and Abe and Hajishengallis were devised to simplify the technical act of placing the ligature[33,36]. Unfortunately, the former protocol requires specialized 3D-printed equipment and possess the potential for premature ligature loss, thereby increasing animal use and the costs associated with additional time spent in the operating room. Furthermore, both protocols generate only small regions of the diseased periodontium available for a study.

The advantages that lie with this technique are grounded in the simultaneous study of oral dysbiosis and immunology that govern the periodontium, utilization of low-cost animals with diverse genetic backgrounds, and simple housing and husbandry practices. As such, goals should be to maximize volumes of diseased tissue and, in attempts to practice the principles of reduction in animal research, reduce animal consumption to a level as low as possible. This requires ensuring that all animals are capable of being included in experimental analyses[37]. However, it should be noted that no matter which animal model of periodontal disease is utilized, there is no single model that encompasses every element of human PD pathophysiology.

This new protocol employs the placement of a ligature around multiple maxillary molar teeth using instrumentation and materials that are found within most laboratories. It allows a

sufficient amount of time to easily and confidently install a ligature that is unlikely to avulse prematurely. Finallu, as PMNs coordinate destruction of the periodontium in PD, a novel methodology to recover oral neutrophils in a manner analogous to humans is also presented.

PROTOCOL:

All murine studies complied with the relevant ethical regulations and were approved by the University of Toronto Animal Care Committee and the Research Ethics Board (Protocol 20011930).

1. Ligature installation

NOTE: This is a non-sterile surgical procedure that can be carried out in a standard operating theater. The use of germ-free animals (not covered here) mandates handling within a biosafety cabinet, use of sterile instruments, and inoculation of the oral cavity with periodontal pathogens to cause the clinical manifestations of periodontitis.

1.1. Administer intraperitoneal anesthesia to 8–12 week-old male C57BL/6 mice, which should be acclimated to their housing facility, using a 0.5" 26 G sterile hypodermic needle and 1 mL syringe according to approved institutional animal care and use committee (IACUC) guidelines.

NOTE: Intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) rapidly and reliably induce anesthesia that lasts for approximately 2 h.

1.2. Assess the anesthetic depth prior to and every 15 min during the procedure as evidenced by loss of the pedal reflex.

1.3. Position the mouse on a heated surgical platform (**Figure 1A)**. Stabilize the maxilla and mandible in the open position using elastic bands and prop the neck with a cotton roll to help maintain the maxilla in a more horizontal orientation (**Figure 1B**). Cover the body and tail of the animal to mitigate heat loss during the procedure.

1.4. Position the surgical microscope at the desired magnification and lighting (if not mounted directly to the microscope) over the oral cavity for visualization of the dentition. While the desired magnification is operator-dependent, optimal visualization of the oral cavity is obtained at 16x.

1.5. Install a sterile 5-0 braided silk suture around the first (M1) and second (M2) maxillary molars within the gingival sulcus using splinter forceps.

NOTE: Braided silk sutures of a smaller gauge can be used for this purpose to facilitate faster installation and reduce the possibility of iatrogenic soft tissue damage.

1.5.1. Position the distal tail of the suture on the palatal side of the dentition and insert the

proximal segment between the contact of M2 and M3 **(Figure 2A)**.

1.5.2. Wrap the suture around the buccal surface of M2 and insert it between the contact of M1 and M2. Ensure that both ends of the suture are pulled tightly to drive the suture into the gingival sulcus and remove all slack **(Figure 2B)**.

1.5.3. Wrap the proximal suture segment around M1, below its height of the contour, and insert it between the contact of M1 and M2. Pull the proximal tail of the suture tightly to drive the suture into the gingival sulcus and remove all slack **(Figure 2C)**.

NOTE: If resistance is observed when inserting the suture between M1 and M2 or M2 and M3, the contact may be open slightly using a standard dental explorer.

1.5.4. Tie the ends of the suture with a surgeon's knot and trim it as close as possible to the knot. Place the knot in the gingival embrasure between M1 and M2 on the palatal side of the maxillary dentition **(Figure 2D).**

NOTE: Steps 2.4.1–2.4.4 can be repeated on the contralateral side, if required.

1.5.5. Clean the surgical instruments in a hot glass bead sterilizer between each animal subject.

CAUTION: The tips of the forceps are extremely sharp and can easily cause oral trauma and significant bleeding. Prepare small segments of gauze to remove blood from the oral cavity and apply pressure to actively bleeding wounds.

1.6. After the ligature installation, remove the mouse from the surgical apparatus, place into a clean cage under a heat lamp and monitor until fully recovered.

1.7. Individually house each mouse with the appropriate environmental enrichment and allow *ad libitum* access to filtered water and mashed standard chow in a temperature- and humidity-controlled environment (12-h light/12-h dark cycle) for 7–11 days.

NOTE: Mashed chow decreases the forces required for mastication, reducing pain associated with mastication, and aids in preventing premature loss of the installed ligature.

2. Sample collection

2.1. Euthanize mice according to the approved IACUC guidelines.

NOTE: Individual euthanasia using a CO_2 chamber followed by cervical dislocation is the preferred method for this protocol. This may be altered depending on experiments that require harvesting of additional tissues.

2.2. Using a pipette, immediately rinse the oral cavity with 100 µL of sterilized 4 ˚C 1x

phosphate-buffered saline (PBS) without calcium and magnesium for 10 s.

2.3. Repeat step 3.2 2x and place each rinse in a single 15 mL conical polypropylene sterile test tube.

2.4. Transfer contents to a 50 mL conical polypropylene sterile test tube by running them through a 40 µm nylon mesh filter.

2.5. Transfer these contents into a new 15 mL conical polypropylene sterile test tube.

NOTE: The final transfer of the sample to a smaller tube allows for the improved visualization of the cellular pellet in the upcoming steps.

2.5.1. If desired, remove the molar ligature(s) and place into 300 µL of sterilized 1x PBS (4 ˚C, without calcium and magnesium) in a separate 15 mL conical polypropylene sterile test tube. Agitate gently and remove the suture from the tube. This sample is treated identically to the oral rinse sample from this point forward.

2.6. Add 33.3 µL of 16% paraformaldehyde (PFA) to facilitate the sample fixation.

2.7. Vortex the samples immediately and incubate on ice for 15 min.

2.8. Fill the tube to 15 mL with 1x PBS to dilute PFA, then centrifuge at 1000 x *g* and 4 ˚C for 5 min.

2.9. Aspirate the supernatant and resuspend the pellet in 1 mL of fluorescence-activated cell sorting (FACS) buffer at 4 ˚C. Count cells on a hemocytometer or automated cell counter.

2.10. Centrifuge the sample again at 1000 RCF and 4 ˚C for 5 min.

2.11. Aspirate the supernatant and resuspend the pellet in an appropriate volume of FACS buffer for a final concentration of 0.5–1.0 x 10^6 cells/50 µL of FACS buffer.

3. Antibody staining for flow cytometric analysis

NOTE: Label and chill all required FACS tubes prior to use.

3.1. Add 1 µL of rat serum and 2 µL of anti-mouse IgG antibody to 50 µL of the sample, vortex immediately, and block on ice for 20 min.

3.2. Add the appropriate antibodies to each sample, vortex immediately, and incubate on ice for 30 min in the dark.

NOTE: Selection and volumes of antibodies depend on prior optimization pilot experiments

tailored to this specific technique.

3.3. Wash samples with 1 mL of FACS buffer, vortexing briefly and centrifuging for 5 min at 1000 *x g* and 4 ˚C. Repeat this step 2x.

3.4. Resuspend samples in 250 µL of FACS buffer, cover tubes with paraffin film, wrap in aluminum foil, and hold at 4 ˚C until analysis.

NOTE: It is ideal to analyze samples within hours of completing the protocol due to the loss of fluorescent signal during long periods of storage. However, samples may be analyzed 2–3 days later if absolutely required.

REPRESENTATIVE RESULTS:

Representative flow cytometry data from oral rinse samples of a naive (**Figure 3A**) and inflamed (**Figure 3B**) murine oral cavity secondary to the ligature-induced periodontitis are provided. Recovery of PMNs from an installed ligature is also demonstrated **(Figure 3C).** Flow cytometer channel voltages were calibrated manually, and compensation was performed with single-stained compensation beads. PMNs were defined as $Ly6G^{+ve}F4/80^{-ve}$ using the outlined gating strategy[38]. A minimum of 500 gated PMN events were acquired from each oral rinse and ligature sample. PMN viability was determined to be approximately 37% by trypan blue staining. Representative images of alveolar bone levels as measured from the alveolar bone crest (ABC) to the cementoenamel junction (CEJ) for healthy (**Figure 4A)** and ligated mice (**Figure 4B)**. The relative differences between these measurements (**Figure 4C**) are provided.

FIGURE AND TABLE LEGENDS:

Figure 1: Animal positioning for molar ligation. (**A**) Access to the oral cavity is achieved by holding the mandible in a depressed position by placing mild traction on maxillary and mandibular incisor dentition. (**B**) Gauze is placed under the skull to prevent movement of the head and to place the maxilla in a relatively horizontal position. The body and tail are covered to prevent heat loss prior to moving the surgical stage under the microscope.

Figure 2: Sequential photograph of ligature installation. (**A**) The distal tail of the suture is positioned on the palatal side of the dentition, and the proximal segment is inserted between the contact of M2 and M3. (**B**) The suture is then wrapped around the buccal surface of M2 then inserted between the contact of M1 and M2. (**C**) The proximal suture segment is wrapped around M1 below its height of contour then inserted between the contact of M1 and M2. (**D**) The ends of the suture are tied with a surgeon's knot and trimmed as close as possible to the knot. The knot is placed in the gingival embrasure between M1 and M2 on the palatal side of the maxillary dentition. All photographs were acquired on dissected maxilla for recording procedural steps with an unobstructed view of the molar dentition.

Figure 3: Representative FACS plots and gating strategy demonstrating oral neutrophil presence. Neutrophils were recovered from the following samples and assessed by flow

cytometry: (**A**) oral rinse from a naïve mouse, (**B**) oral rinse, and (**C**) recovered ligatures from a mouse with ligature-induced periodontitis (bilaterally). Representative gating strategy scatterplots are shown. Singlets (SSC-H x SSC-W and FSC-H x FSC-W) and neutrophils ($Ly6G^{+ve}/F480^{-ve}$) were gated as shown. Numerical values reflect the percentage of cells within each gate. SSC-A = side scatter area; SSC-W = side scatter width; SSC-H = side scatter height; FSC-A = forward scatter area; FSC-W = forward scatter width; FSC-H = forward scatter height.

Figure 4: Alveolar bone loss differences between control and ligated animals. Representative photos demonstrating differences in alveolar bone loss between (**A**) control and (**B**) ligated mice. (**C**) Measurements from the cementoenamel junction (CEJ) to alveolar bone crest (ABC), along with the mesiopalatal and distopalatal line angles of M1 and M2, are shown (mean ± SEM, n = 3). P-values were determined by two-way ANOVA with a post-hoc Fisher's LSD test. (*$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$).

DISCUSSION:

The most critical element associated with use of the murine ligature-induced model of periodontitis is centered around the retention of the ligature until the time of sacrifice or intentional removal. The installed biofilm-retentive ligature is capable of inducing a significant loss of alveolar bone height in as few as 6 days, plateauing between the 11–16 day period[39]. The decision to sacrifice animal subjects before the maximal period of bone loss, rendering this a much shorter model of ligature-induced periodontitis, was selected to further reduce the incidence of premature ligature avulsion, defined as the loss of the ligature prior to the time of sacrifice, which renders the animal nondiagnostic.

The most commonly cited disadvantage of this model highlights the perceived technical difficulty of installing the ligature due to the miniscule dimensions of murine molar dentition, which range in circumference from 4.4 mm (M1) to 2.6 mm (M3)[40]. This issue can be mitigated through the involvement of surgical staff/trainees for ligature installation, which requires (at most) 10 min/animal and includes the delivery of anesthesia. Furthermore, placement of the ligature is a skill mastered through repetition, and it is no more intensive than many common lab-based techniques that require an elevated degree of manual dexterity. From a procedural standpoint, the use of intraperitoneal anesthesia provides extensive time to facilitate ligature installation. If required, it also allows replacement of any ligatures that are not installed in a satisfactory manner, as the localization of the suture to the gingival sulcus and knot between the contact of M1 and M2 is ideal for retention.

The proposed modifications of these protocols, which call for either 1) ligation of a single molar using a circumferential suture or 2) a single linear segment of suture placed between a single contact of the molar dentition, provides several advantages. From a technical perspective, the protocol utilizes equipment that is either readily available or can be constructed from pre-existing equipment in a similar manner to Abe and Hajishengallis. This obviates the need for special orders or manufacturing of equipment by 3D printing[33]. The installation of a continuous silk suture sling around multiple teeth secured with a surgeon's knot that cannot be disturbed

by the tongue may also improve ligature retention.

During the course of this protocol, compared to the technique proposed by Marchesan et al. in which losses of up to 20% are anticipated, there was a complete absence of premature ligature avulsion[36]. While the incidence of ligature loss was not directly assessed by Abe and Hajishengallis, the technique has been applied in a number of studies[41–44]. From a technical perspective, iatrogenic soft tissue damage was avoided in ligated animals. Factors contributing to this phenomenon include a continuous observation of the oral cavity through a microscope, constant visualization of the tips of forceps, and use of clean forceps to prevent slipping off of the suture during placement.

Within the oral cavity, the elongated segments of suture material also dramatically increase the amount of diseased tissue available for analysis and can result in a marked decrease in animal use. This is because all subjects are able to be included in analysis, and pooling of tissue and rinse samples is not required. It should be noted that it is possible to ligate the contralateral maxillary molar dentition utilizing the previously reported techniques to increase the affected tissue area[41]. However, this modification is not possible in cases where a split-mouth design is required, lending support for the application of this new protocol. Finally, akin to the previous ligature-induced periodontitis protocols, use of this technique is not limited to 8–12 week-old male C57BL/6 mice. Mice of older age, either sex, and various genetic backgrounds may be acceptable depending on experimental design.

Unfortunately, this model still possesses some technical and methodologic faults. The use of specific pathogen-free mice as detailed above cannot mimic the progression of human periodontitis. This is due to several differences between the composition of their commensal oral biofilms, which limits the external validity of evaluating bacteria-bacteria and bacteria-host interactions[45]. This can theoretically be mitigated through the use of germ-free mice, which complicates the protocol significantly. Additional efforts and resources are required to ensure that animal subjects remain germ-free during the housing and surgical manipulation. Further measures must also be instituted to develop periodontitis, as these mice are resistant to ligature-induced periodontitis in the absence of bacterial accumulation[46,47].

Furthermore, mono-infection and co-infection ligature models fall short of recapitulating the human condition, as periodontal disease represents a more complex polymicrobial interaction between the biofilm and host immune system. In these circumstances, elucidating the roles of, and interactions between a limited number of human oral bacteria may be considered deficient and potentially disadvantageous, especially in the context of studying periodontium immunobiology.

Finally, recent evidence has implicated that the forces of mastication are capable of modifying gingival immunosurveillance through the accumulation of T helper 17 cells, an integral mediator of barrier immunity[48]. As such, the use of a soft diet, especially in older animals, may act a potential confounder. This may reduce the potential for the normal physiologic bone loss in regions where ligatures have been applied. In light of this evidence, careful consideration

should be given to the use of aged animals and appropriate age-matched controls where these findings have been noted to be most prominent.

The use of this relatively simple model can be extended to evaluating alveolar bone loss by micro-computed tomography, histologic analyses of both the alveolar bone and surrounding attached gingiva, and conducting oral microbiota characterization, all of which have been accomplished with the existing ligature-induced PD models[33,49]. Although rarely discussed or utilized, it is also feasible to assess the effects of treatment, (as well as the repair and regeneration of the periodontium) in the setting of ligature-induced murine periodontitis by removing the ligature under general anesthesia with fine-point splinter forceps[39]. Finally, use of this model may be extended to studying the systemic effects of periodontal disease due to the increased inflammatory load caused by the ligation of more than one tooth, as well as the left and right maxillary molar dentition, if desired.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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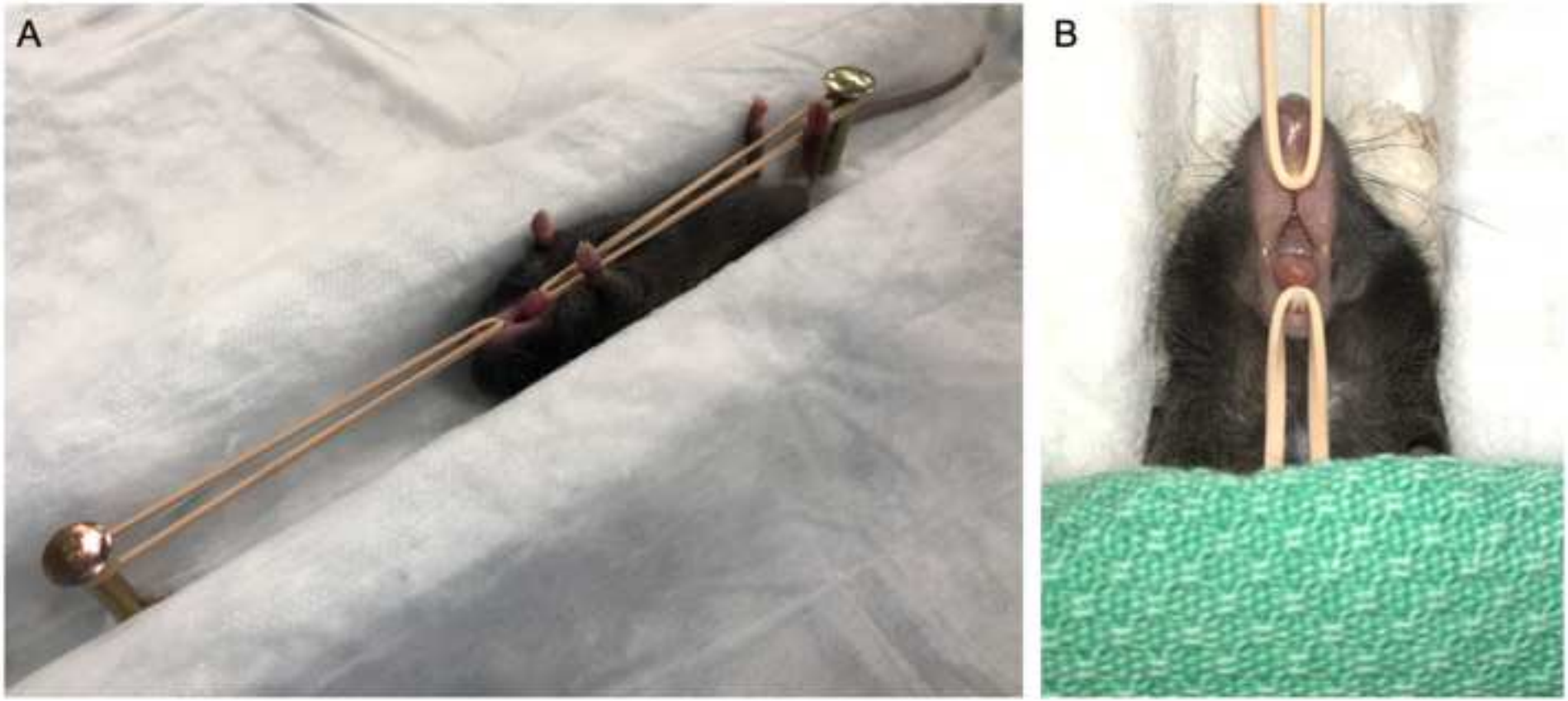

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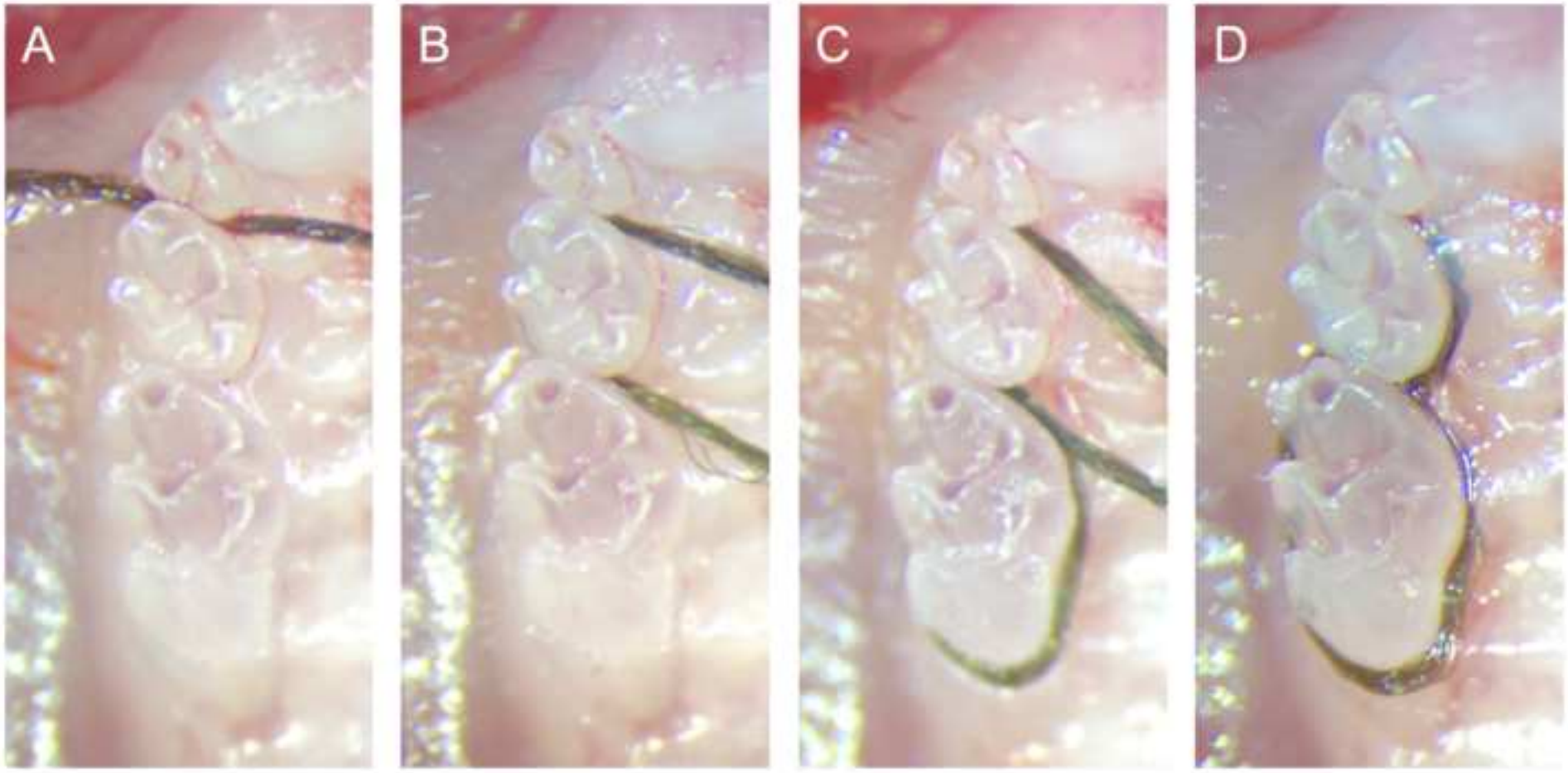

Figure 3

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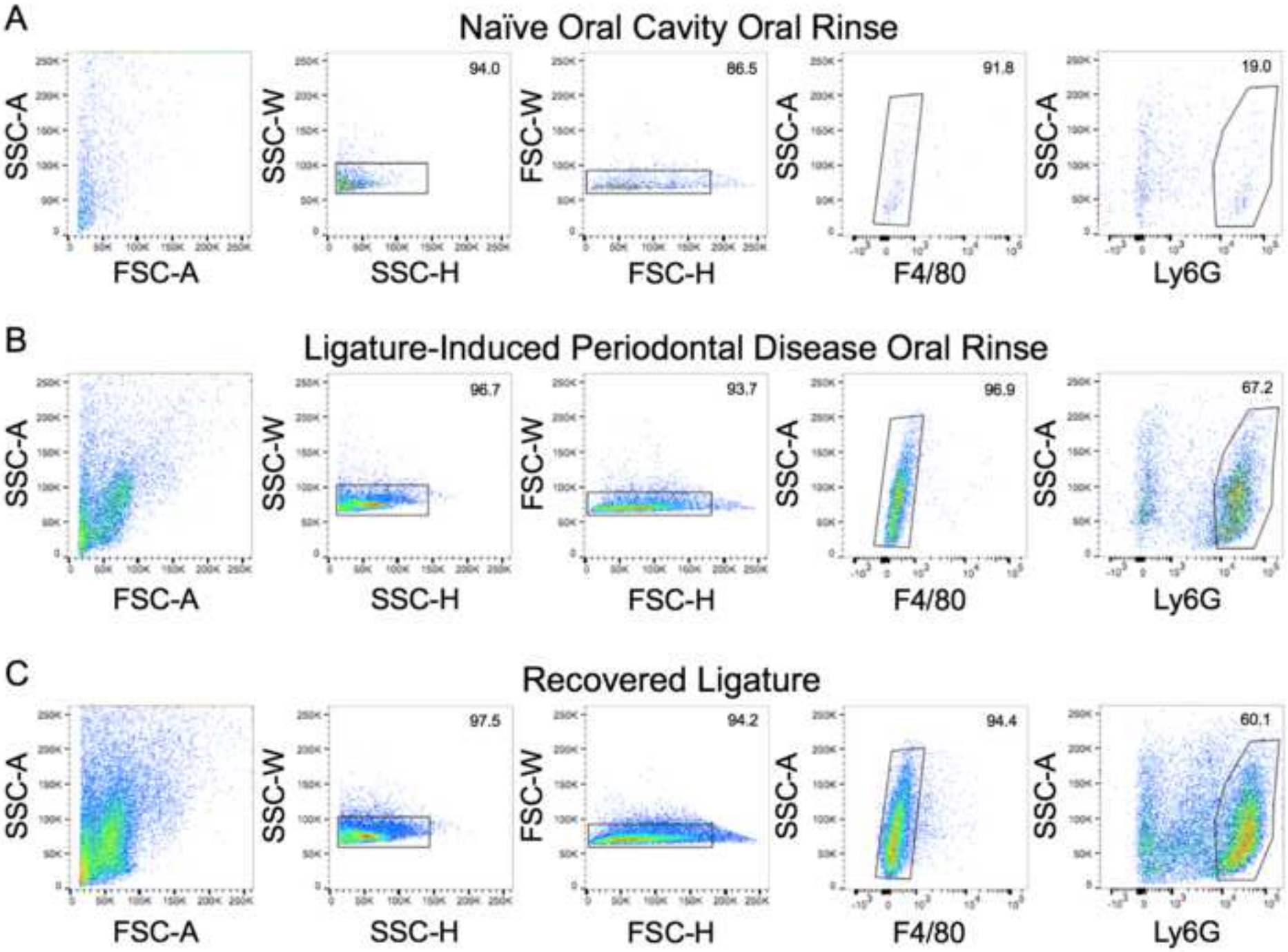
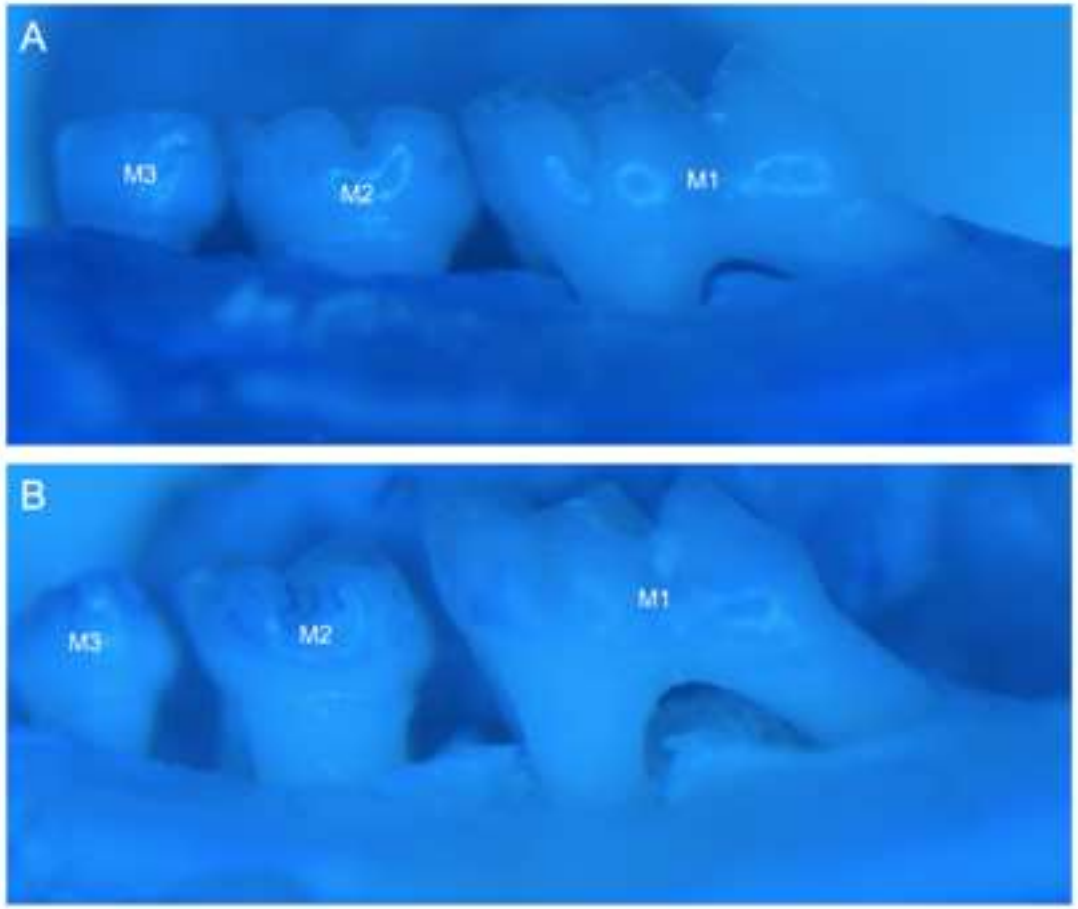


Figure 4



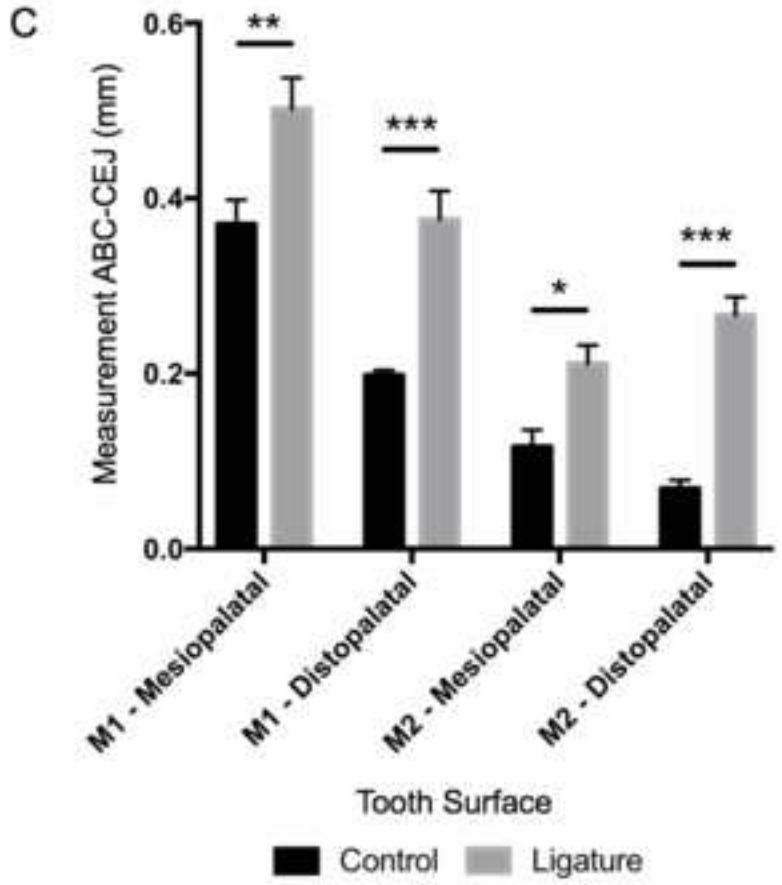


Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Anti-mouse F4/80 Antibody	BioLegend	123131
Anti-mouse Ly6G Antibody	BD	560602
C57BL/6 Male Mice	Charles River	
Conical Centrifuge Tube	FroggaBio	TB15-500
Conical Centrifuge Tube	FroggaBio	TB50-500
FACS Buffer	Multiple	
FACSDiva	BD	
Fibre-Lite	Dolan-Jenner	
FlowJo	Tree Star	
Heat Therapy Pump	Hallowell	
Hot Glass Bead Sterilizer	Electron Microscopy Sciences	66118-10
Iris Scissors	Almedic	7602-A8-684
Ketamine	Vetoquinol	
LSRFortessa	BD	
Mouse Serum	Sigma	M5905-5ML
Nylon Mesh Filter	Fisher Scientific	22-363-547
Paraformaldehyde	Fisher Scientific	28908
Phosphate-buffered Saline	Sigma	D1408-500ML
Plastic Disposable Syringes	BD	309659
Rat Serum	Sigma	R9759-5ML
Silk Suture	Covidien	SS652
Splinter Forceps	Almedic	7726-A10-700
Splinter Forceps	Almedic	7727-A10-704
Stereo Dissecting Microscope	Carl Zeiss	28865
Sterile Hypodemic Needle	BD	305111
Syringe	BD	309659
Xylazine	Rompun	

Comments/Description
BV421, Clone BM8
PerCP-Cy5.5, Clone 1A8
8 to 12 weeks old
15 mL
50 mL
1% BSA (BioShop), 2mM EDTA (Merck), 1x $HBSS^{-/-}$ (Gibco)
v8.0.1
Model 180
v10.0.8r1
HTP-1500
Germinator 500
Straight
100mg/mL
X-20
40 µm
16% (w/v), Methanol Free
Without $CaCl_2$ and $MgCl_2$, 10x
1 mL
C13 USP 5-0
#1
#5
Photo-Zusatz
26G X 1/2"
1 mL
20mg/mL

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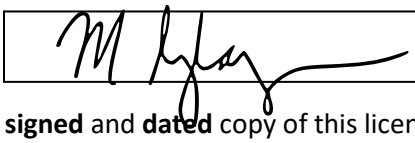
Signature:	
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12. 2.1: Do you check the depth of anesthesia?

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13. 2.3: What is the desired magnification?

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14. 4.4: Please provide FACS gating strategy.

Figure 2 outlines the FACS gating strategy for oral cavity samples from naïve and ligated mice as well as recovered ligatures. Please let the authors know if an alternative presentation of this information is required.

15. Once done please highlight 2.75 pages of the protocol including headings and spacings that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

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20. Please alphabetically sort the materials table.

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Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The present study proposes a modification of ligature induced periodontitis (LIP) described by Abe & Hajishengallis (1) associated with a novel oral rinse protocol to recover and study neutrophils. The modification proposed, was made with the idea of increase the area of inflammation and bone loss together with avoid the premature avulsion of the ligature. The associated method of murine oral rinse, in my view an original idea and innovation for the oral mucosal research, provides a clever approach to study neutrophils in the oral cavity.

Major Concerns:
1. In my opinion, the authors expended too much time explaining and justifying the modification made to the LIP rather than focusing in the novel and smart approach of the oral rinse.

Acknowledged and corrected in the manuscript. The need for selecting an appropriate model of LIP is paramount to the design of any investigation which the authors feel justifies the length of time spent exploring the details of these techniques.

2. Authors also justify this modification based on several limitations of a technique described by Marchesan et al. (2) but also include the optimized LIP described by Abe & Hajishengallis (1) as part of these problems. They should state very clear in the manuscript that these are 2 very different techniques and the shortcomings of one procedure (2), can't be applied to the other (1). The optimized LIP procedure, as was described by Abe & Hajishengallis (1), has been used in numerous studies (3-6), demonstrating to be a very reliable and relative simple procedure for the study of the pathological events that occur during periodontitis.

Acknowledged and corrected in the manuscript.

3. The previously optimized LIP technique (1), involves equipment available in any laboratory and doesn't need special orders or 3D printed materials, which was stated as an advantage of the modified technique described in this article.

Acknowledged and corrected in the manuscript.

4. Another stated advantage of this modified LIP is the absence of ligature avulsion, and again this comparison can only be made with the technique described by Marchesan et al. (2). There is no evidence of avulsion in the optimized LIP technique (1) and it is not demonstrated that the lingual knot could affect the retention of the ligature.

The authors agree with the observation that this statement can only be applied to the technique described by Marchesan et al. and this has been amended in the manuscript. This phenomenon was, unfortunately, not directly addressed by Abe and Hajishengallis. The role of the lingual knot has been minimized.

5. A final advantage of the modification of the LIP stated by the authors is the increased area of diseased tissue obtained. In my opinion, this statement or conclusion is not supported in this article. The authors must demonstrate that using their LIP they obtained more diseased tissue compared to the other

techniques. Moreover, if one of the goals is to reduce the numbers of mice, you could ligate the molars of both maxilla (right and left) as was showed recently (6).

The act of bilateral ligation was added to the manuscript as an option for only those individuals who are not performing split-mouth studies.

6. Authors should report the rate of oral trauma that they normally have performing this technique (Lines 197-199). Is important to report and comment that because wounds produce inflammation and repair. Those processes could modify the numbers of neutrophils recovered during the rinse procedure described. Moreover, an open and bleeding wound could contaminate with blood neutrophils the sample of cells obtained from the rinse.

Acknowledged and completed (see manuscript). There were zero instances of oral trauma in the hands of our operator. The authors, however, recognize this concern. The most likely timing and location for such an event would be as follows:

1. During the tying of the knot and associated with the palate if the forceps lost hold of the suture or during its placement
2. During ligature placement associated with the maxillary buccal vestibules

The eyes of the operator should be directed through the microscope and tips of the instruments should be kept visible at all times to help avoid this complication.

Minor Concerns:

7. In the abstract says that the variants of LIP are isolated to focal regions and subject to premature avulsion of the installed ligature (line 42). Please specify in the main text what is premature for the authors.

Acknowledged and added to manuscript. Premature would be defined as the loss of a ligature prior to sacrifice which in this case was seven days.

8. In 2.2, authors should include a picture of how they positioned the mice (line 162-165). In my opinion a picture can help the readers to understand how to stabilize the maxilla and mandible.

Acknowledged and added to manuscript.

9. Authors should comment on how the use of mashed chow (2.6; line 205) could modify the results obtained with this technique. A recent publication (7) demonstrate that forces of mastication could influence in bone loss.

This is an excellent observation and would certainly be relevant if the application of a mashed diet was used in aged animals. The data supporting the association of mechanical forces influencing bone loss was not as robust for young animals which were used in this protocol. Discussion related to this paper was, however, added in a cautionary manner. Further, animal care committees may encourage the use of mashed chow to limit pain associated with mastication which, in those cases where an inflammatory process is under study, can be markedly altered through the administration of several common analgesics.

10. The fixation of the cells with PFA before the surface staining could affect epitopes and decrease the

number of surface markers that can be used. In a time when flow cytometry is expanding their capability to detect more that 20 markers in a cell, how this could affect to the procedure?

The authors understand this concern which was recently addressed in the following manuscript:

Fine N, Barzilay O, Sun C, Wellappuli N, Tanwir F, Chadwick JW, et al. 2019. Primed PMNs in healthy mouse and human circulation are first responders during acute inflammation. *Blood Adv*. 3(10):1622–37.

The selection of epitopes, for the purpose of reporting this technique, were merely used to identify PMNs within the oral cavity leaving it to the readership to explore the various panels that could be applied for the study of innate oral immunology.

11. References 33 and 44 are the same references.

Reference section corrected.

References:
1. Abe T, Hajishengallis G. Optimization of the ligature-induced periodontitis model in mice. J Immunol Methods. 2013;394(1-2):49-54.
2. Marchesan J, Girnary MS, Jing L, Miao MZ, Zhang S, Sun L, et al. An experimental murine model to study periodontitis. Nat Protoc. 2018;13(10):2247-67.
3. Dutzan N, Kajikawa T, Abusleme L, Greenwell-Wild T, Zuazo CE, Ikeuchi T, et al. A dysbiotic microbiome triggers TH17 cells to mediate oral mucosal immunopathology in mice and humans. Sci Transl Med. 2018;10(463).
4. Eskan MA, Jotwani R, Abe T, Chmelar J, Lim JH, Liang S, et al. The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss. Nat Immunol. 2012;13(5):465-73.
5. Hiyari S, Wong RL, Yaghsezian A, Naghibi A, Tetradis S, Camargo PM, et al. Ligature-induced peri-implantitis and periodontitis in mice. J Clin Periodontol. 2018;45(1):89-99.
6. Tsukasaki M, Komatsu N, Nagashima K, Nitta T, Pluemsakunthai W, Shukunami C, et al. Host defense against oral microbiota by bone-damaging T cells. Nat Commun. 2018;9(1):701.
7. Dutzan N, Abusleme L, Bridgeman H, Greenwell-Wild T, Zangerle-Murray T, Fife ME, et al. On-going Mechanical Damage from Mastication Drives Homeostatic Th17 Cell Responses at the Oral Barrier. Immunity. 2017;46(1):133-47.

Reviewer #2:
Manuscript Summary:
The manuscript outlines a model of periodontitis in mice. The model presented is a development of the current ligature model already used by numerous groups, but presents new ways to increase disease severity as well as monitoring characteristics of immune cells within the oral rinse. As such this model is important to share with the research community. However a few clarifications are required before publication.

Major Concerns:
1) The authors should include some time lines/information on the length of time before bone loss is seen and/or until euthanasia. Is this a shorter model that the standard ligature Periodontitis model or is it the same?

This is a shorter model supported by the following paper which characterizes the earliest period where significant alveolar bone loss has manifested: Viniegra, A., Goldberg, H., Çil, Ç., Fine, N., Sheikh, Z., Galli, M., et al. Resolving Macrophages Counter Osteolysis by Anabolic Actions on Bone Cells. *J Dent Res*. 97 (10), 1160–1169 (2018).

2) Importantly, data should be included detailing the level of bone loss that will be seen post ligature placement (for eg ABC-CEJ or bone volume changes). This is important for any reader to appropriately understand whether they are carrying out the model correctly. At the moment no representative results detailing disease severity are included.

Acknowledged and added to manuscript.

3) In Figure 2 where representative FACS data is shown, the authors should include information on cell viability; ideally a live/dead FACS stain. Again this is important for any reader so that they can get an idea of whether their isolation of cells is the same as the authors.

Acknowledged and added to manuscript.

Minor Concerns:
1) The reference by Abe and Hajishengallis is included twice; as ref 33 and 44.

Reference section corrected.

Reviewer #3:
Manuscript Summary:
This manuscript nicely describes and illustrates an improved method to induce periodontitis in a mouse model with the use of ligatures.

Major Concerns:
none

Minor Concerns:
It would improve the manuscript if the advantages of the nicely illustrated method were better emphasized.

Acknowledged and discussion modified.