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Systematic Scoring Analysis for Intestinal Inflammation in a Murine Dextran Sodium Sulfate-Induced Colitis Model

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TITLE:

Systematic Scoring Analysis for Intestinal Inflammation in a Murine Dextran Sodium Sulfate-Induced Colitis Model

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KEYWORDS:

DSS-colitis, inflammation, injury, erosion, ulcer, histological colitis score

SUMMARY:

Systematic scoring of intestinal inflammation using a free computer-assisted system is a powerful tool to quantitatively compare histopathological changes in colitis models characterized by the presence of ulcers and inflammatory changes. Histological colitis score evaluation strengthens clinical observations and facilitates data interpretation.

ABSTRACT:

Murine colitis models are tools that are extensively employed in studies focused on understanding the pathobiology of inflammatory intestinal disorders. However, robust standards for objective and reproducible quantification of disease severity remain to be defined. Most colitis analysis methods rely on limited histological scoring of small segments of intestine, leading to partial or biased analyses. Here, we combine high-resolution image acquisition and longitudinal analysis of the entire colon to quantify intestinal injury and ulceration in the dextran sodium sulfate (DSS) induced model of murine colitis. This protocol allows for the generation of objective and reproducible results without extensive user training. Here, we provide

comprehensive details on sample preparation and image analysis using examples of data from DSS induced colitis. This method can be easily adapted to other models of murine colitis that have significant inflammation associated with mucosal injury. We demonstrate that the fraction of inflamed/injured and eroded/ulcerated mucosa relative to the complete length of the colon closely parallels clinical findings such as weight loss amid DSS-induced disease progression. This histological protocol provides a reliable time and cost-effective aid to standardize analyses of disease activity in an unbiased way in DSS colitis experiments.

INTRODUCTION:

The gastrointestinal epithelial barrier plays a pivotal role in separating luminal antigens and pathogens from underlying tissue compartments[1]. Epithelial injury and mucosal wounds seen in pathologic conditions such as inflammatory bowel disease (IBD), ischemia, or surgical injury are associated with clinical symptoms that include diarrhea, weight loss, blood in stool, and abdominal pain. In response to injury, epithelial cells migrate and proliferate to re-epithelialize and repair mucosal barrier defects. Resolution of inflammation and restitution of mucosal integrity are crucial to re-establishing intestinal mucosal homeostasis and function[2-4].

Various animal models have been employed to study the underlying molecular mechanisms that are associated with the damage to the intestinal epithelial barrier. Well-established and easily applicable models of chemically induced colitis are widely used, particularly in studies related to inflammatory injury such as IBD. A common, reproducible, and reliable murine colitis model employs dextran sodium sulfate (DSS) mediated colonic injury and inflammation. The severity of disease varies depending upon mouse strain, dose of DSS, length of DSS administration, and molecular weight of DSS[5-7].

Intestinal mucosal damage during DSS colitis is usually evaluated using the Disease Activity Index (DAI), a composite score determined by weight loss, fecal blood content, and stool consistency. The fecal blood content can be microscopic (detected using a stool guaiac acid test) or macroscopic; fecal consistency is classified as hard, soft, or liquid (i.e., diarrhea)[5,8]. Scoring of these clinical parameters can be subjective and may vary depending on the user's experience and bias, although overall, the data provides reliable information and is thus widely used by IBD researchers. In contrast, there is no generally accepted method for histological evaluation of mucosal damage. Most commonly, selected areas of the colon are inspected by a trained pathologist and scored based on several parameters that usually include crypt injury and leukocyte infiltration[9-11]. However, because the number of investigated parameters and the amount of tissue analyzed varies considerably between individual reports, comparability of many published studies is limited. To reduce observer bias and enhance inter-study concordance, an ideal histological scoring protocol should: 1) include the entire length of the colon, as intestinal mucosal inflammation is most often variable and skip lesions are common, 2) limit analysis to specific key and easily interpretable parameters to reduce subjectivity, 3) facilitate fast, consistent processing of large numbers of samples, and 4) use widely available and affordable tools for data acquisition, analysis and presentation.

Here we describe a technique to process the entire colon or long segments of the small intestine

in a “Swiss roll” configuration along with the use of a free computer-assisted scoring system to analyze intestinal mucosal inflammation and damage because of DSS-induced colitis.

PROTOCOL:

All animal experiments described were approved by the University of Michigan's Committee on the Use and Care of Animals.

1. Tissue harvest

1.1. Euthanize mice humanely using isoflurane anesthesia followed by cervical dislocation, in accordance with approved protocols. For all animal experiments, approval was obtained by a certified review board in accordance with the National and Institutional guidelines for animal handling.

1.2. Place the mouse on a dissecting pad in a supine position. Immobilize mouse extremities using 20 G x 1 1/2-inch G needles.

1.3. Using forceps and scissors, make a small incision on the abdominal skin and pull it to the side to expose the peritoneum.

1.4. Open the abdominal cavity with a midline incision in the peritoneum from the pubic bone to the sides of the abdomen.

1.5. Carefully remove tissues and organs until large intestine is visualized. Cut the pelvic bone on both sides of the colon to fully visualize the organ, extending from the anus towards the cecum.

1.6. Gently remove fat, small veins and arteries attached to the colon, while carefully dissecting the organ, cutting just proximal to the anus and just distal to the cecum.

NOTE: The dissected colon should remain at room temperature while completing the Swiss roll procedure.

1.7. Carefully flush the colon with 1x PBS, using a flexible plastic gavage needle inserted through the anus to remove fecal contents.

1.8. Position the colon in a straight line, and open longitudinally along the mesenteric artery. Bisect the colon longitudinally from the distal to the proximal end (**Figure 1A**). One half of the tissue can be used for histological analysis while the other can be processed for western blot, PCR or rolled into a second Swiss roll for fresh frozen immunofluorescence microscopy[12].

2. Preparation of swiss rolls

2.1 Trim extra tissue from the proximal colon using a razor blade until approximately the

same width along the length of the whole colon is obtained.

2.2 Align the colon to expose the lumen facing-up, and flatten the tissue completely using a flexible gavage needle. Add more PBS, if needed, to keep the tissue moist throughout the procedure.

2.3 Remove the excess of PBS using a paper wipe. With a syringe and gavage needle, add 10% neutral buffered formalin solution over the tissue for 2-3 min to fix and flatten the tissue.

2.4 Use straight forceps to grab the end of the distal colon and twist the colon into concentric circles from the distal to proximal end (**Figure 1B**).

NOTE: It is possible to push back the inside of the Swiss roll while rolling using the forefinger to ensure the tissue is inside the roll.

2.5 Insert a 27 G needle to pin the colon in the middle to hold its Swiss roll shape (**Figure 1C**).

2.6 Place the Swiss roll with the needle into an embedding cassette inside a histologic specimen container.

NOTE: Tissue must be oriented in parallel with respect to the cassette before fixation (**Figure 1D**).

2.7 Fix the tissue in 10% neutral buffered formalin solution overnight at 4 ˚C[13].

2.8 After overnight fixation, wash the tissue 3x with PBS.

2.9 Add 70% ethanol to the tissue prior to the paraffin embedding process. Remove the needle from the Swiss roll before proceeding. Tissue can be stored in ethanol at room temperature until paraffin embedment[13].

2.10 Place samples into the tissue processor, embed in paraffin, and prepare 4 µm sections, mounted on positively charged microscopy slides (**Figure 1E**). This can be an optional stop point.

NOTE: Proper tissue orientation is critical to achieve sections suitable for image analysis. Parallel Swiss roll embedding in the paraffin cassette will result in full sections appropriated for image analysis (**Figure 1F-1H**). Oblique sections must be avoided to prevent incomplete sections (**Figure 1I-1K**). For more details, see the Discussion section.

2.11 Stain sections with hematoxylin and eosin (H&E)[13].

3. Digital scanning and analysis

NOTE: For accurate evaluation of mucosal changes, select only sections that include at least 90% of the total colon length.

3.1 Scan stained sections using a slide scanner or imager (see **Table of Materials**). Images produced need a resolution of 0.25 microns per pixel with 40x objective and 40x magnification.

3.2 Install and download an appropriate software for digital analysis of scanned slides (see **Table of Materials**).

3.3 Open scanned images in the image processing software (**Figure 2A**). Verify that the entire colon is visible and that there are no missing areas of the sample.

3.4 Activate the label imager and scale bar tools to properly identify scanned slides by clicking **Label imager** (**Figure 2A**).

3.5 Open the annotations tool by clicking **Annotations**, (**Figure 2A**) and create 3 different layers by clicking **New layer** (**Figure 2B**) to quantify the total length the Swiss roll, inflammation/injury, and erosion/ulceration. Choose a different color for each layer by clicking **Layer color** (**Figure 2B**).

3.6 Measure the length of each layer/category by clicking the **Pen tool** (**Figure 2A**), using the muscularis mucosa as a reference:

3.6.1. View the image at 400 µm zoom (or more) to facilitate adequate visualization of the muscularis mucosa.

NOTE: Magnification is easily controlled using the mouse scroll wheel and will need to be adjusted as necessary while moving across the section to draw all the lines.

3.6.2. Click on the **Pen** tool to draw a line following the muscularis mucosa (**Figure 2A**). Move the pointer as needed to visualize the adjacent area for analysis.

NOTE: Each time that the pen is stopped, a small new layer region will be generated. It can be visualized and edited with the **Layer regions** tab (select desired segment and click **Delete Layer** in case of mistakes or corrections, **Figure 2B**).

3.7 Once all layers (**Figure 2C**) are defined, export the data using the **Export Grid to Text File** button inside the layer regions options (**Figure 2B**).

NOTE: Save files often while creating the layers to ensure data is properly stored.

3.8 Open the text files and copy the data with a spreadsheet software. Total all the segments from each region and calculate percentage of injury and ulceration with respect to total length.

3.9 To calculate the Histological Colitis Score (HCS) and to evaluate the severity of disease, consider three main characteristics as detailed below.

3.9.1 Check for healthy intestinal mucosa which is characterized by organized epithelial cells in the crypt-luminal axis, lamina propria with few immune cells, and subjacent muscularis mucosa that interfaces the mucosa and submucosa (**Figure 3A**).

3.9.2 Check for inflammation/injury which is characterized by epithelial crypts that are attenuated or partially missing epithelial cells and mucosal inflammation with neutrophil infiltration into crypts (**Figure 3B**).

3.9.3 Check for the presence of erosion/ulceration which is characterized by areas devoid of surface epithelium or areas completely lacking epithelial crypts with or without associated leukocytes (**Figure 3C**).

3.10. Calculate HCS of injured and ulcerated regions expressed as a percentage of the total length in the following formula:

$$HCS = \frac{\%inflammation/injury + 2 * \%erosion/ulceration}{10}$$

NOTE: HCS combines the percentage of inflammation/injury and erosion/ulceration adding a factor of two to the latter, based on a reasonable assumption that complete loss of the epithelium results in maximum loss of barrier integrity and hence worse disease. HCS consistently represents the morphological changes caused by DSS induced experimental colitis. Interestingly, we have not seen a clear correlation between the numbers of colonic lymphoid aggregates or follicles and clinical disease severity in DSS colitis and, therefore, we did not include the quantification in this analysis.

3.11. Take a snapshot of the representative images (click **Snapshot**, **Figure 2A**), and save. Include scale bars if needed by clicking on **Show/Hide Scale Bar** (**Figure 2A**).

REPRESENTATIVE RESULTS:

To illustrate the reliability of this Histological Colitis Score Analysis in the context of mucosal damage after DSS challenge and subsequent recovery from colitis, we administered 2.5% DSS in the drinking water of eight 10 weeks old male C57BL6 wild type mice for 5 days followed by a recovery period with regular water for 5 days. There was no change in body weight during the acute administration of DSS, from day 0 to 5 (**Figure 4A**). Body weight dramatically decreased after day 5, when mice started drinking regular tap water. Blood and soft stools appeared after 3 days of DSS administration and continued for the subsequent 3 days on water until day 8 of the experiment (**Figure 4B**). These observations suggested that the most detrimental effects of exposure to DSS were observed during the recovery period between days 5 and 8. Measurement of the colon length on the day 10 confirmed a significant shortening at the end of the experiment (**Figure 4C**). Colons were harvested at days 0, 2, 5, 7, 8, and 10 to make paraffin Swiss rolls. Tissue was stained with H&E and high-definition scans (**Figure 4D**) were analyzed to calculate the Histological Colitis Score (**Figure 4E**) and percentage of injury and ulceration (**Figure 4F**).

Figure 4D shows normal crypt architecture in untreated C576BL6 mice, superficial to the muscularis mucosa and supported by the lamina propria (day 0, arrow). After 2 days of DSS administration, immune cell recruitment was observed (day 2, arrow). On day 5, epithelial cells appeared damaged, and there was an infiltration of neutrophils across the epithelium (cryptitis) associated with epithelial injury (day 5, arrow). From day 5 to 8, areas of epithelial loss with inflammation and ulceration were noted (day 7 and 8, arrows). The latter is commonly observed as the mice drink regular tap water during the recovery phase. Finally, epithelial cells begin to regenerate and repopulate ulcerated areas, slowly restoring colonic mucosa at day 10 (arrow).

HCS closely mirrors simple assessment of percentage of inflammation/injury and erosion/ulceration, and both quantitatively demonstrate that there is significant damage to the colon of mice during recovery from DSS on day 5 to 10, which correlates with epithelial erosions and leukocyte infiltration shown in **Figure 4D**. Data provided by HCS distinguishes between the severity of the disease with regards to inflammation associated with epithelial injury versus erosion/ulceration.

These observations demonstrate that the systematic scoring system of HCS proposed in this protocol constitutes a reliable tool to quantify mucosal damage.

FIGURE AND TABLE LEGENDS:

Figure 1: Sample preparation is crucial for accurate data analysis. (**A**) Distal colon fully extended and opened along the mesenteric artery. (**B**) Swiss rolling process. (**C**) Needle insertion to hold Swiss roll shape (**D**) paraffin embedding cassette. (**E**) Paraffin block. (**F**) Swiss roll parallel orientation to avoid partial sections. (**G**) Complete Swiss roll, scale bar: 2 mm. (**H**) Ideal section of colonic crypts, scale bar: 80 µm. (**I**) Oblique Swiss roll orientation. (**J**) Incomplete Swiss roll, scale bar: 2mm. (**K**) Oblique section of colonic crypts, scale bar: 80 µm.

Figure 2: Quantitative evaluation of mucosal damage. (**A**) Basic tool bar with Snapshot, zoom slider, show/hide scale bar/axes/grid, label imager, annotations, and pen tool features, among others. (**B**) Annotations menu displaying layer color, new layer, delete layer, show/hide layer, delete region and export grid to text file buttons. (**C**) Overview of total length, injured and ulcerated layers, scale bar: 3 mm.

Figure 3: Morphology of mouse intestinal mucosa stained with hematoxylin & eosin after acute DSS-experimental colitis followed by recovery. (**A**) Healthy intestinal cells organized in colonic crypts surrounded by lamina propria and separated from the submucosa by the muscularis mucosa, scale bar: 200 µm. (**B**) Acute mucosal inflammation or injury with infiltration of neutrophils into the mucosa and crypt epithelium, associated with crypt epithelial distortion/loss and epithelial attenuation, scale bar: 200 µm. (**C**) Ulcerated or eroded areas with complete epithelial loss and associated inflammation., scale bar: 200 µm.

Figure 4: Histological Colitis Score and percentage of injury and ulceration correlates with body

weight and stool index during DSS experimental colitis: (**A**) Body weight and (**B**) stool indexes are evaluated daily in wild type mice treated with 2.5% DSS for 5 days (red line), followed by 5 days of recovery on water (black line). Data are representative of two independent experiments with at least 4 mice per group and are expressed as mean ± SEM. Significance is determined by two-way ANOVA and Sidak multiple comparison, *p<0.033, **p<0.002, and ***p<0.001. (**C**) Colon length of mice was measured at day 10. Data are representative of two independent experiments with at least 3 mice per group and are expressed as mean ± SEM. Significance is determined by two-tailed Student's t test, ***p<0.001. (**D**) Hematoxylin and eosin (H&E)-stained tissue sections of colonic mucosa were analyzed to calculate, scale bars: 60 μm. (**E**) Histological Colitis Score (HCS) and percentage of injury/ulceration relative to the length of the colon. Data are representative of two experiments with 2 mice per group and are expressed as mean ± SEM. Significance is determined by one-way ANOVA and Tukey multiple comparison, *p<0.033, **p<0.002, and ***p<0.001.

DISCUSSION:

Our Histological Colitis Score system constitutes a reliable tool to quantify tissue inflammation and damage in the intestine. This approach provides an improved understanding of the histopathological state of the whole organ without the bias of selecting small areas or incomplete sections. Among the critical steps to successfully execute this protocol are proper preparation of Swiss rolls that allow for analysis of at least 90% of the length of the colon; parallel orientation during paraffin embedding and sectioning to ensure straight and uniform tissue sections with longitudinal views of epithelial crypts; practice and training with supervision from an experienced pathologist to confirm that trainees identify the differences between acute inflammation, epithelial injury, and erosion/ulceration; and access to a high resolution digital scanner.

Small adjustments and troubleshooting are required to appropriately orient the Swiss roll during paraffin embedding as well as during sectioning to ensure that the tissue is parallel oriented relative to the paraffin cassette and blade (**Figure 1E-F**). Adhering to this spatial orientation will reduce the number of incomplete sections. If the colon is perfectly rolled on itself, the best sections including most of the tissue length will be located near the middle of the Swiss roll (**Figure 1F**). Collecting several sections greatly increases the chance of obtaining well-oriented sections where the entire length of the crypts is visible (**Figure 1G-H**). Avoid sectioning oblique Swiss rolls (**Figure 1I-J**), characterized by circular-shaped crypts or crypt donuts (**Figure 1K**). Often visualization of the sections collected under the microscope is strongly recommended to ensure proper technique. A good section captures the entire length of the colon.

Any instrument that enables to capture the entire length of the Swiss roll and provides the resolution needed to magnify the tissue to visualize the crypt architecture is adequate for evaluation of the HCS. Some microscopes allow the overlap of small images and reconstruct entire tissue sections. However, the resolution of these images might be poor after increasing magnification to score the tissue. Additionally, the overlap of sections may not be perfect, leaving areas of tissue that are not well defined and impossible to accurately score.

Among the advantages of the scoring protocol proposed here are the classification of the tissue

in only two categories inflammation/injury or erosion/ulceration. This simplifies and accelerates the classification of tissue damage, while increasing reproducibility. The software used for the analysis is accessible, free, and easy to use. HCS accurately reflects mucosal damage, as it allows for quantifying ulcerated areas along the entire length of the colon and appropriately provides more weight towards this pathology over inflamed, non-ulcerated regions. This is clearly reflected when the HCS data is compared versus percentage of injury/ulceration (**Figure 4E-F**). Scoring analysis of each sample takes approximately 40 minutes. With the appropriate training, access to scanned slides, a computer, and the software, most researchers can perform the analysis. However, it is always advisable to confirm scoring results with a pathologist to ensure accuracy and reproducibility. Assessment of intestinal inflammation can be performed with any software that allows high resolution magnification of slides and length measurements; however, for simplicity, in this protocol we focused on the software listed in the **Table of Materials**.

We have been using this scoring system to successfully analyze disease across various DSS colitis experiments. For example, Kelm et al.[14] explored the importance of glycosylation of CD44v6, an important regulator of epithelial migration and proliferation, using a novel antibody (GM35). They demonstrated reduced disease activity index scoring and improved HSC in mice challenged with acute DSS following targeting of sialyl lewis A glycans on epithelial CD44v6, confirming the importance of post translational modification of CD44 variants for recovery after DSS induced injury[14]. In another example, Reed et al.[15] used a chronic DSS model to study mucosal injury and repair in mice with an epithelial specific deletion of CD47, a transmembrane protein important for inflammatory and repair processes. Colon scans were analyzed and scored to calculate the percentage of injury and ulceration as described above, demonstrating markedly impaired mucosal repair in mice lacking epithelial CD47[15]. The chronic colitis model in this report employed cyclical administration of DSS to analyze injury and repair over time.

Additional publications from our group and others have demonstrated the advantages of this scoring system and its correlation with clinical symptoms[6,16,17]. This protocol describes in detail how to prepare and score Swiss rolls, using free and easily accessible software. The simplicity of the scoring proposed here is based on use of only two categories (inflammation/injury or erosion/ulceration) in scanned slides to facilitate the scoring process and increase reproducibility. Importantly, the scoring system described here also correlates with clinical parameters of intestinal inflammation. In conclusion, the Histological Colitis Score is a tool that can be used to quantitatively score DSS-colitis and can be applied to other colitis models associated with inflammation and epithelial injury/loss.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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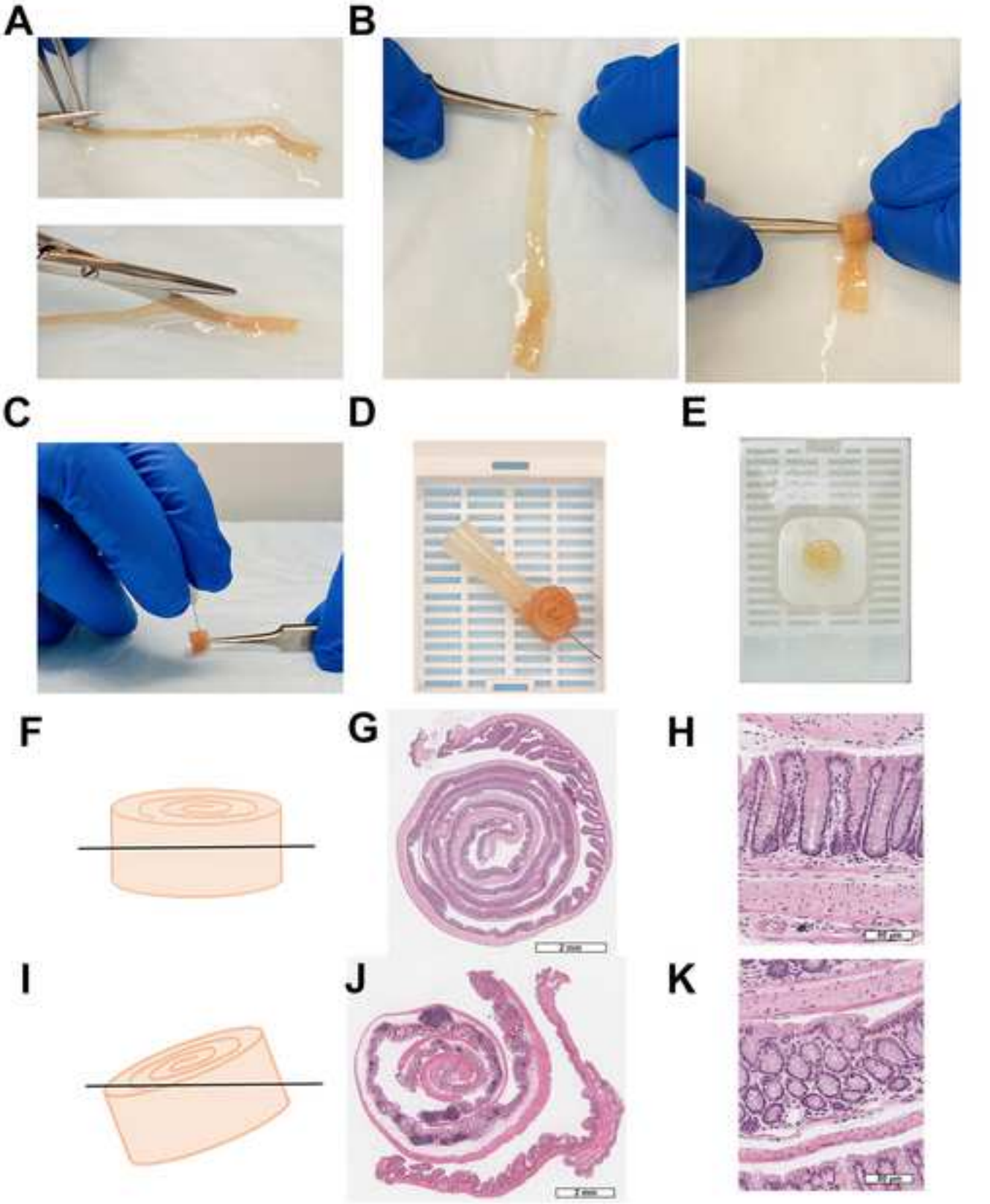

Figure 1

Figure 2

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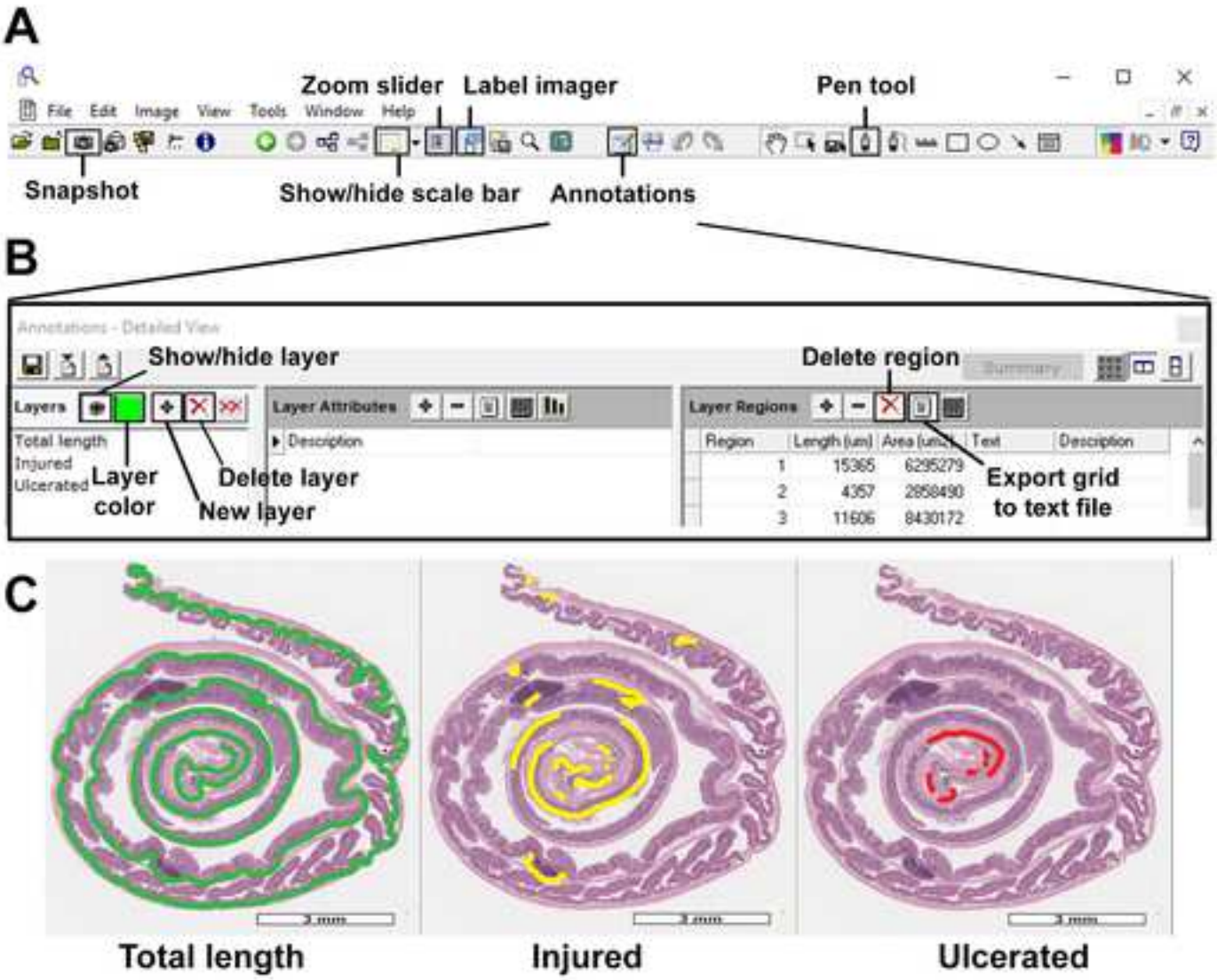

Figure 2

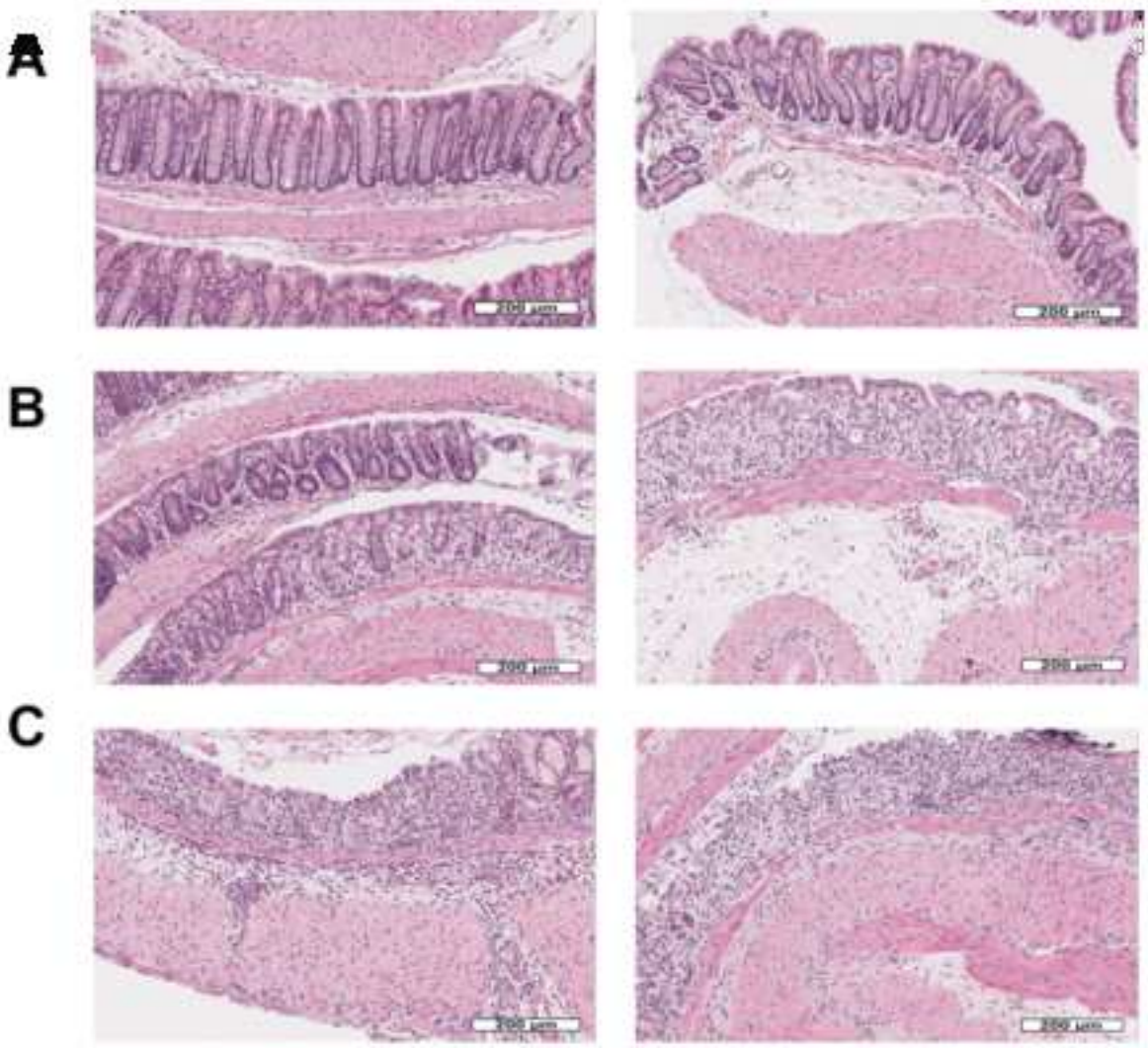

Figure 3

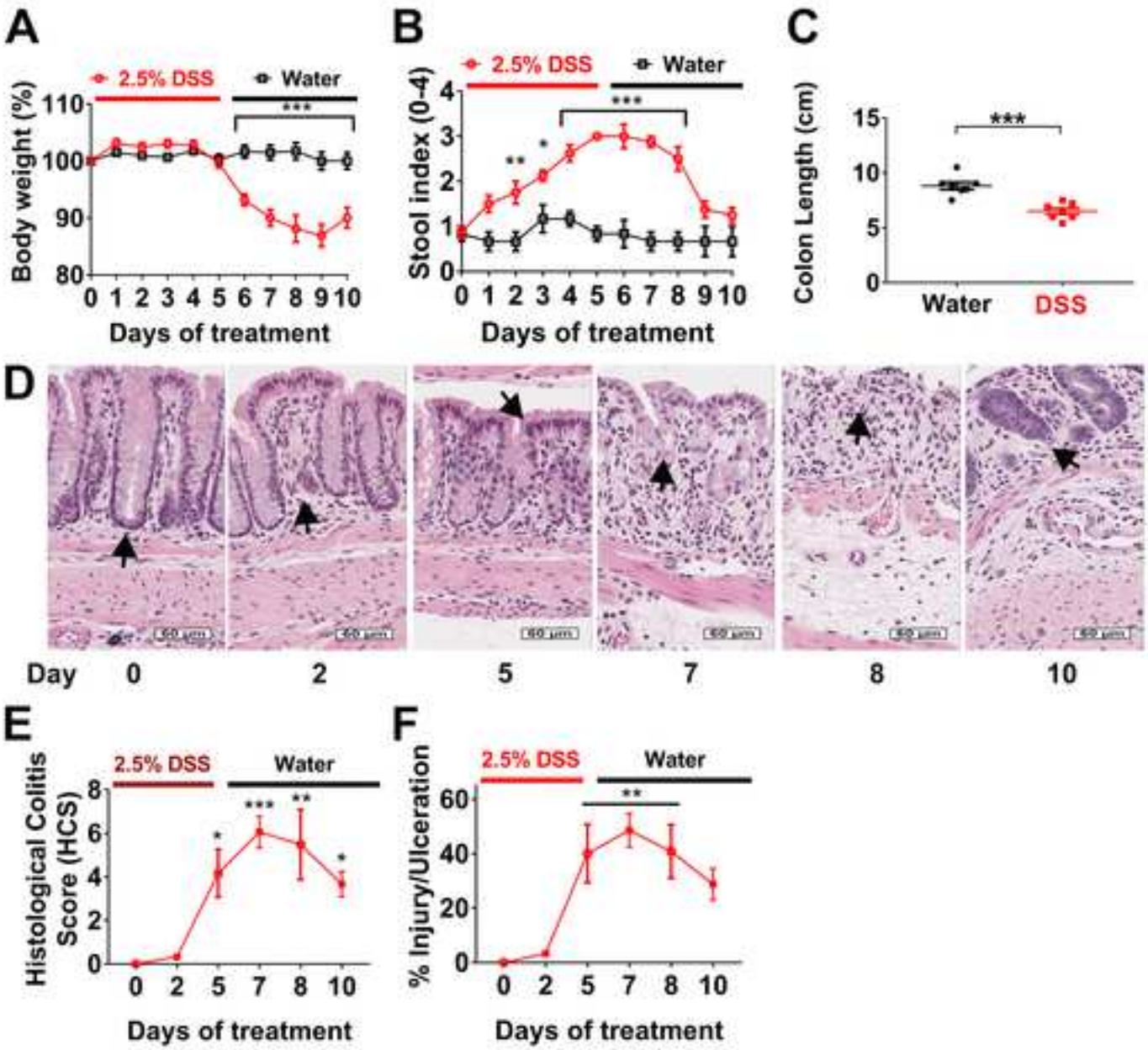

Figure 4

Name of Material/ Equipment	Company	Catalog Number
Aperio AT2 – High Volume, Digital Whole Slide Scanning	Leica Biosystems	Aperio AT2
Absorbent Underpads with waterproof moisture barrier	VWR International	56616-032
American Line 66-0089 Single Edge Blade, 100 per pkg	GT Midwest	TL5837
BD Luer-Lok Disposable Syringes without Needles	Fisher scientific	14-823-2A
Bonn Strabismus Scissors - ToughCut	Fine Science tools	11103-09
Bonn Strabismus Scissors - ToughCut	Fine Science tools	14084-09
Dumont #5 Forceps	Fine Science tools	11251-20
Formalin solution, neutral buffered, 10%	Sigma	HT501640-19L
HistoPrep 70% Ea	Fisherbran	70% denatured ethyl alcohol Version
ImageScope	Aperio	12.3.3.5039
LeakBuster Specimen Containers: Sterile	Starplex Scientific	B120210
Phosphate-Buffere Saline, without calcium & magnesium	Corning	21-040-CV
Plastic Feeding tubes, 20 GA x 30 mm	Instech	FTP2030
PrecisionGlide Needle, Size: 20 G x 1 1/2 in	BD (Becton, Dickinson and Company)	305176
PrecisionGlide Needle, Size: 27 G x 1/2 in	BD (Becton, Dickinson and Company)	305109
Syringe, 10 ml	BD (Becton, Dickinson and Company)	302995
Unisette Tissue Cassettes	Simport	M505-2

Comments/Description

http://www.leicabiosystems.com/pathology-imaging/aperio-epathology/integrate/imagescope/

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Title: "An Unbiased Approach to Analyze Intestinal Inflammation in a Murine Dextran Sodium Sulfate-Induce Colitis Model".

We would like to thank the reviewers for their comments. Below is a point-by-point response to the reviewer's comments. Additional information and discussion points are included in the revised manuscript and are highlighted in red color.

Response to the reviewer's concerns:

Reviewer #1:

This manuscript describes a method to accurately and reproducibly measure histological damage in colonic tissue from mouse models of colitis. This is important, as the authors state, since assessment of histological damage is reported using various methods, is subjective, and since investigators often use segments of colon rather than the entire length, is open to sampling bias. Standardization and simplification of the method would be very helpful to investigators.

Response: Thank you for this positive comment.

1. The authors should indicate the temperature that should be used for steps 1.7 to 2.2. Should the tissue be kept on ice or is room temperature ok?

Response: The dissected colon should remain at room temperature while completing the Swiss roll procedure. This statement has been added to section 1.6 of the revised manuscript.

2. The authors refer to "perpendicular sections" in 2.10 (Figure 1I-J). It is more accurate to say that this example is an oblique section.

Response: As recommended, we have changed this sentence to "oblique section" in section 2.10, figure legend 1I, and the discussion.

3. It would be helpful if the authors were to indicate that the Aperio ImageScope software is free the first time it is mentioned (line 179). Readers might assume this is an expensive analysis program and be turned off at the outset.

Response: Thank you for this comment. We now mention this in the summary and at the end of the introduction.

4. Is it safe to assume that the method can be used on regular histological micrographs (is a digital scanner necessary)? This could be discussed.

Response: Thank you for this comment. We added the paragraph in the discussion section.

Any instrument that enables to capture the entire length of the Swiss roll and provides the resolution needed to magnify the tissue to visualize the crypt architecture is adequate for evaluation of the Histological Colitis Score. Some microscopes allow the overlap of small images and reconstruct entire tissue sections. However, the resolution of these images might be poor after increasing magnification to score the tissue. Additionally, the overlap of sections may not be perfect, leaving areas of tissue that are not well defined and impossible to accurately score.

Reviewer #2: The sampling and pathological evaluation of samples taken from animals at autopsy is very underestimated issue. It can importantly contribute to irreproducibility of the study results. Authors expose that for accurate interpretation of damage caused in the colon, the whole length of colon needs to be histologically evaluated to get more reliable results. I agree with authors opinion. Authors thus demonstrated step by step description how to perform Swiss role method (which is already known) and histologically evaluate slides with the help of the computer program. Authors also provide one formula and propose that such evaluation can be done by researchers and not pathologists.

1) Please be aware that DSS model do not develops ulcers but erosions - lesions do not penetrate muscularis mucosa - so please use histologicaly adequate terminology.

Response: We agree that erosions represent superficial injury while ulcers are associated with deeper injury. However, in DSS colitis, we think it is fair to qualify areas of complete loss of epithelial crypts with inflammation extending into and through the muscularis mucosa as severe erosions or "ulcers". To better illustrate the inflammation and injury in DSS we now provide higher magnification images in figure 3 showing inflammation extending into the muscularis mucosa as well as areas of crypt loss in the distal colon in acute DSS colitis. However, the reviewers point is well taken and there are also many areas with more superficial epithelial loss that clearly represent simple erosions. We now refer to such areas as erosion/ulceration to avoid confusion.

2) Line 117: Cutting colon next to the anus - cutting colon from abdominal cavity without cutting the pelvis is a subject of bias

Response: We cut the pelvic bone in order to dissect the colon. We now include this information in section 1.5 of the revised manuscript.

3) Where and how authors get the formula how to calculate histological colitis score? Please explain? Having a formula without any explanation why erosion needs to be multiplied by factor of two and not three or four is a bit unprofessional.

Response: We respectfully take odds with the comment that our scoring proposal is unprofessional. In many characterizations of histopathology, scoring has been used to signify disease or not, or mild, moderate, or severe disease. While such scoring can be viewed as arbitrary since the exact contributions of each parameter is not necessarily specific, we have proposed a simple scoring system that places more weight on erosion/ulcer than mucosal inflammation/injury based on a reasonable assumption that complete loss of the epithelium results in maximum loss of barrier integrity, for which we have arbitrarily assigned a values of 2x compared to injury alone. True, we don't know what the exact contributions to disease morbidity are, this scoring system allows for consistent and reproducible data representation that mirrors % inflammation/injury and erosion/ulceration as highlighted in Fig 4F. We have now added a comment on our rationale for numerical assessment in section 3.9.

4) Lesions and the severity of lesions or development of inflammation or erosion depend on the timepoint when study ends. As it was demonstrated in the paper, in first days we can see damage and few inflammatory cells, following by inflammation and few days later erosions develop. Several days or weeks after induction of the

disease we can see selrecovery or in case of progression of the disease we can see chronic inflammation of different severity and not erosions. Also inflammation can range from mild to severe, which significantly affect the health state, but author did not evaluate this issue. Near the anus we can obsere also squomase metaplasia. How to evaluate this lesion?

Response: We agree that the inflammatory response and injury depend on the time point of when the study ends. However, in our hands and highlighted in this manuscript, clinical disease severity most closely correlates with mucosal inflammation/injury and erosion/ulceration. This is the simple point we are making and have added comments to better highlight this association in section 3.9. Regarding squamous metaplasia, we have not observed this in acute DSS colitis, and the squamous epithelium is naturally present in the distal rectum near the anus.

5) Authors did not propose what to do with the Payers patches or lymph nodes that are usually present in the normal colon as well as injured colon.

Response: Peyer's patches are localized to the small intestine where as in the colon collections of lymphocytes are referred to as lymphoid aggregates (LA) or isolated lymphoid follicles (ILFs). We have not seen a clear correlation of between the numbers of LA/ILFs and clinical disease severity in DSS colitis. Indeed, the number of ILFs can vary significantly between mice raised in different animal facilities. We comment now comment on this in the revised manuscript on section 3.9.

6) I do not agree with the authors that evaluation of the histology can be done by researchers and not pathologists - According to my opinion the main problem in research is that samples are not evaluated by pathologist but scientist or researcher - I have seen many papers that reported transmural inflammation and demonstrate transmural inflammation with figures -but on figure there was Payer patch and not transmural inflammation. There can also be technical artefacts on the slides, which can be recognized and correctly interpreted only by experienced pathologists but not by researcher. And if there will appear new pathology it will be unrecognized and missed by a researcher, which is again unprofessional and does not contribute to validity and quality of the scientific work and justification of animals used. Please reconsider the consequences of such statements.

Response: Two of us are experienced GI pathologists and, as indicated in the manuscript, we have proposed this protocol for generation of objective and reproducible data since current methodologies rely on limited histologic scoring of small segments of the intestine. Importantly, we demonstrate that the fraction of ulcerated and injured mucosa relative to the complete length of the colon closely parallels clinical findings such as weight loss amid DSS-induced disease progression. We believe these are simple parameters that can be easily and widely adapted in the research community. However, we appreciate the reviewer's comments and have tempered our statements and added a statement encouraging pathologist interpretation and researcher education in using this scoring method in the fourth paragraph of the discussion section.

Respectfully,

Asma Nusrat, MD

Charles A. Parkos, MD, PhD

Vicky Garcia-Hernandez, PhD

December 14th, 2020

Manuscript Reference: JoVE62135

Dear Dr. Bajaj,

Thank you for providing us an opportunity to resubmit a revised version of our manuscript entitled "An Unbiased Approach to Analyze Intestinal Inflammation in a Murine Dextran Sodium Sulfate-Induce Colitis Model".

Below is a point-by-point response to the editors' comments, and attached is a separate document with a point-by-point response to the reviewers' comments. We would like to point out that some of the reviewer 2 comments are not professional and incorrect. For example, we disagree with reviewer 2 comment pertaining to ulcer vs. erosion and presence of Peyer's patches or lymph nodes in the colon. Peyer's patches are seen in the small intestine and the colon has few lymphoid aggregates that are not lymph nodes. We have responded to reviewer 2 regarding the comment related to ulcer vs. erosion. Two of the authors on this manuscript (Parkos and Nusrat) are practicing gastrointestinal pathologists with deep understanding of human disease as well as mouse colitis models.

All the additional information and discussion points are included in the revised manuscript and are highlighted in red color.

Response to editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We have proofread and corrected the manuscript. Abbreviations are now defined at first use.

2. To avoid abbreviations in the title, please revise your title to "An Unbiased Approach to Analyze Intestinal Inflammation in a Murine Dextran Sodium Sulfate-Induced Colitis Model".

Response: As recommended we have not used abbreviations in the title.

3. Please provide an email address for each author.

Response: Email addresses are now included for each author.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Aperio AT2 scanner, Leica Aperio ImageScope, Aperio software etc.

Response: We have edited our manuscript to comply with JoVE's publication requirements.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: We have edited our manuscript accordingly.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We have edited our manuscript accordingly.

7. Please specify what strain, age, and sex of mice were used. Also, please specify the method of euthanasia without highlighting it.

Response: We have provided more information about the number, sex, strain, and age of the mice used (line 244). The euthanasia method was clarified in step 1.1.

8. 1.8: Please cite references for histological preparation and analysis for western blotting and PCR.

Response: As requested, a histological preparation reference was added in sections 2.7 and 2.9. We do not have any western blot or PCR data in this manuscript. However, we added a reference for more details about these techniques in section 1.8.

References:

1. Luna, L. G., Armed Forces Institute of Pathology (U.S.) & Armed Forces Institute of Pathology (U.S.). Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3d edn, (Blakiston Division, 1968).
2. Flemming, S. *et al.* Desmocollin-2 promotes intestinal mucosal repair by controlling integrin-dependent cell adhesion and migration. *Molecular Biology of the Cell.* 31 (6), 407-418, (2020).

9. 2.10: Please move the note explaining Figure 1's panels with respect to optimal and less than optimal results to the representative results.

Response: Consistent with comment #14, this paragraph is more appropriate for the discussion section as it is directly related to the critical steps and troubleshooting.

10. 2.11: Please cite references for H&E staining.

Response: A reference was added to section 2.11.

Reference:

1. Luna, L. G., Armed Forces Institute of Pathology (U.S.) & Armed Forces Institute of Pathology (U.S.). Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3d edn (Blakiston Division, 1968).

11. 3.2, 3.3: Please remove reference to commercial products and the url. Instead please use generic language like "Use appropriate software for digital analysis of scanned slides (see Table of Materials)" and enter information about Leica ImageScope software and the url in the Table of Materials.

Response: References to the commercial software and the url were removed from our manuscript (section 3.1 and 3.2, and 3.3).

12. Please revise Figure 2's legend title to "Quantitative evaluation of mucosal damage".

Response: The figure legend was modified as requested.

13. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legends.

Response: Scale bars and appropriate figure legends were added and modified in all the figures using ImageScope software.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Response: In response to the editor and Reviewer #1 suggestions, we have added the following two paragraphs in the discussion section of the revised manuscript:

1. Small adjustments and troubleshooting are required to appropriately orient the Swiss roll during paraffin embedding as well as during sectioning to ensure that the tissue is parallel oriented relative to the paraffin cassette and blade (Figure 1E-F). Adhering to this spatial orientation will reduce the number of incomplete sections. If the colon is perfectly rolled on "itself", the best sections including most of the tissue length will be located near the middle of the Swiss roll (figure 1F). Collecting several sections greatly increases the chance of obtaining well-oriented sections where the entire length of the crypts is visible (Figure 1G-H). Avoid sectioning oblique Swiss rolls (Figure 1I-J), characterized by circular-shaped crypts or crypt "donuts" (Figure 1K). Often visualization of the sections collected under the microscope is strongly recommended to ensure proper technique. A good section captures the entire length of the colon.
2. Any instrument that enables to capture the entire length of the Swiss roll and provides the resolution needed to magnify the tissue to visualize the crypt architecture is adequate for evaluation of the Histological Colitis Score. Some microscopes allow the overlap of small images and reconstruct entire tissue sections. However, the resolution of these images might be poor after increasing magnification to score the tissue. Additionally, the overlap of sections may not be perfect, leaving areas of tissue that are not well defined and impossible to accurately score.

15. Please do not abbreviate journal names in the reference list.

Response: The reference list was modified as requested.

16. Please sort the Materials Table alphabetically by the name of the material.

Response: The Materials Table was modified as requested.

Respectfully,

Asma Nusrat, MD

Charles A. Parkos, MD, PhD

Vicky Garcia-Hernandez, PhD