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Visualization of Estrogen Receptors in the Colon of Mice with TNBS-Induced Crohn's Disease using Immunofluorescence

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FACULTY OF BIOLOGY
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Mr. Benjamin Werth
Senior Science Editor
Journal of Visualized Experiments

Dear Sirs,

In response to your invitation enclosed you will find our manuscript entitled "Visualization of estrogen receptors in the colon of mice with Crohn's disease by immunofluorescence" by Jacenik D., Zielińska M., Michlewska S., Fichna J., Krajewska W.M.. The manuscript has neither been publish nor is currently under consideration for publication either in whole or in part, by any other journal. Submission has been approved by each of co-authors. Authors declare no conflict of interests. We would greatly appreciate if you consider this manuscript for publication in *Journal of Visualized Experiments*. We hope you will find our manuscript interesting and suitable for publication in *JoVE*.

Sincerely yours.

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TITLE:

Visualization of Estrogen Receptors in Colons of Mice with TNBS-Induced Crohn's Disease using Immunofluorescence

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KEYWORDS:

Crohn's disease, murine model, estrogen receptors, G protein-coupled estrogen receptor, GPER, ERα, ERβ, immunohistochemistry, confocal microscopy

SUMMARY:

The protocol presents a complete validated TNBS-induced murine model of Crohn's disease and methods for visualization of estrogen receptors by immunohistochemistry using immunofluorescence of formalin-fixed colon sections embedded in paraffin.

ABSTRACT:

Crohn's disease is the most diagnosed type of inflammatory bowel disease. Chronic inflammation developing in the intestine leads to peristalsis disorder and damage of intestinal mucosa and seems to be associated with an increased risk of colon neoplastic transformation. Accumulating evidence indicates that estrogens and estrogen receptors affect not only hormone-sensitive tissues, but also other tissues not directly related to estrogens, such as the lungs or colon. Here, we describe the protocol for the successful immunofluorescence staining of estrogen receptors in colon obtained from a murine model of TNBS-induced Crohn's disease. A detailed protocol for the induction of Crohn's disease in mice and intestine preparation is provided as well as a step-by-step immunohistochemical procedure using formalin-fixed paraffin-embedded intestine sections. The described methods are not only useful for estrogen receptor detection and estrogen signaling investigation in vivo but can also be applied to for other proteins which may

be involved in the development of colitis in mice.

INTRODUCTION:

Crohn's disease (CD) is an inflammatory bowel disease (IBD) manifested as chronic intestine inflammation. The etiology of CD is poorly understood, but there are a few major factors that appear to be responsible for CD development, including intestinal microbiota, and genetic and environmental factors, such as diet or stress[1]. For a better understanding of the pathogenesis of Crohn's disease, several models of intestinal inflammation have been used[2-7]. In this article, we present results obtained from a 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced murine model of CD.

It has been documented that estrogens are capable of modulating chronic intestinal inflammation[8-12]. The biological activity of estrogens is mediated by cognate receptors, among which are nuclear estrogen receptors (ERs), i.e., ERα (gene *ESR1*) and ERβ (gene *ESR2*), as well as G protein-coupled estrogen receptor, i.e., GPER (gene *GPER1*), referred to as membrane-bound ER[13,14]. There are several methods for determining the level of estrogen receptors, but only a few can be used to visualize them in the intestine.

Immunohistochemistry (IHC) is a widely used method in clinical and basic studies for the detection of certain antigens in cells or tissues with fluorochrome-conjugated antibodies. IHC seems to be an important method in tissue structure visualization, as well as in the identification and localization of specific proteins, which may be crucial for understanding the development of colitis. Here, we present a complete and validated protocol for immunohistochemical visualization of estrogen receptors in the intestine using immunofluorescence.

PROTOCOL:

Animal studies were conducted with the consent of the Local Ethical Committee (28/ŁB29/2016) in accordance with Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, and institutional recommendations.

1. TNBS-induced murine model of Crohn's disease

NOTE: This protocol uses male BALB/C mice weighing 25–28 g. Animals are housed at a constant temperature (22–24 °C) and, relative humidity 55 ± 5%, and maintained in a 12 h light/dark cycle with free access to standard chow pellets and tap water ad libitum.

1.1. Place the mouse into the induction chamber and close the lid tightly. Anesthetize the mouse briefly with isoflurane (25% O_2 with O_2 flow rate at 1.5–2 L/min).

NOTE: Respiratory rate should remain rhythmic and slower than normal and should not change in response to a noxious stimulus.

1.2. Instill 4 mg of TNBS in 0.1 mL of 30% ethanol in 0.9% NaCl or 0.1 mL of 30% ethanol in 0.9% NaCl as a vehicle control into the distal colon through a catheter.

NOTE: The catheter should be carefully introduced approximately 3 cm into the anus.

1.3. Monitor the mouse daily from day two to eight for clinical parameters including body weight, rectal bleeding, stool consistency and mortality.

1.4. On day eight, euthanize the mouse by cervical dislocation.

[Place **Figure 1** here]

2. Separation and macroscopic evaluation of colon

NOTE: One day before colon separation, dilute 100 µL of antibiotic in 1 mL of phosphate buffer saline (PBS) and leave at 4 °C overnight.

2.1. Clean the skin over the abdomen using 75% ethanol and sterile gauze.

2.2. Cut the abdominal wall from breastbone to anus using sterile scissors and tweezers.

2.3. Cut off the colon as close as possible to the anus and cecum.

2.4. Place the colon on the Petri dish. Cut the colon along from the anus into the cecum end. Clean and wash the colon 2–4 times in cold antibiotic-PBS solution.

2.5. Perform macroscopic evaluation according to **Table 1** using a caliper.

[Place **Table 1** here]

NOTE: Tissue adhesion* and erythema/hemorrhage#, fecal blood# and diarrhea# are subject to visual assessment. *Tissue adhesion evaluate using a three-point scale (0: colon without tissue adhesion, 1: colon with moderate tissue adhesion, 2: colon with extensive tissue adhesion); #based on absence (0) or presence (1) of erythema/hemorrhage, fecal blood and diarrhea.

2.6. Convert the length of the ulcer in centimeters to a point scale, i.e., every 0.5 cm of ulcer is counted as 0.5 point. Convert the thickness of the colon in millimeters to a point scale, i.e., every *n* mm corresponds to *n* points.

2.7. Convert the length of the colon in centimeters on a three-point scale. The length of colon obtained from each mouse with Crohn's disease is evaluated in relation to the average colon length for the control group (0: <10% shorter than the control, 1: from 10 to 20% shorter than the control, 2: over 20% shorter then the control).

2.8. Calculate the total macroscopic score according to the equation: Total macroscopic score = adhesion (points) + erythema/hemorrhage (points) + fecal blood (points) + diarrhea (points) +

length of ulcer (points) + colon thickness (points) + colon length (points).

3. Colon sample preparation

3.1. Cut the colon into 1–2 cm fragments and place each on sponge in an appropriately labeled histological cassette.

NOTE: Sponges for histological cassettes prevent colon folding during dehydration and incubation in liquid paraffin.

3.2. Place the colon fragment in 4% formaldehyde and incubate for at least 24 h at 4 °C.

3.3. Prepare and program the tissue processor for 1 h of incubation in 50%, 70%, 90%, 95%, 100% ethanol, xylene/100% ethanol (1:1; v/v), and xylene only, as well as for at least 3 h of incubation in liquid paraffin.

NOTE: Dehydration must be performed in increasing concentrations of ethanol and xylene, but the concentration of ethanol can be modified. The xylene/ethanol mixture is recommended but not required.

3.4. Transfer the colon fragment to a histological box and place in the pre-programmed tissue processor.

3.5. Run the tissue processor.

3.6. After incubation steps, place the colon fragment in a metal mold so that the two ends of the colon are in an upright position and fill one third of the mold with liquid paraffin.

3.7. Place the mold in the cooling area (-5 °C) for a few seconds, and then move the mold to the warming area (70 °C). Place in the bottom part of the histological box and cover the entire colon fragment with liquid paraffin.

3.8. Leave the metal mold with the colon fragment in paraffin for a few minutes in the cooling area. Remove the metal mold from the paraffin block and incubate for at least 24 h at 4 °C.

3.9. Remove excess paraffin from the block and insert it into a fully automated rotary microtome.

NOTE: The paraffin block may be stored at -20 °C for a few minutes before this step.

3.10. Cut the colon fragment into 5 µm sections.

3.11. Transfer the colon section to a water bath preheated to 40 °C.

3.12. Use the labeled glass slide to remove the colon section from the water bath.

NOTE: The colon sections float on the water. Put the labeled glass slide in the water under the colon section and withdraw the glass slide carefully.

3.13. Leave the glass slide for 24 h at room temperature. For long term storage, keep the glass slide at 4 °C after 24 h of incubation at room temperature.

4. Immunohistochemistry with immunofluorescence staining

NOTE: Do not allow the colon section to dry at any step during the procedure.

4.1. Remove paraffin by incubating the glass slide in xylene for 5 min. Repeat this step three times.

4.2. Place the glass slide in xylene/100% ethanol (1:1; v/v) for 5 min. Repeat this step three times.

4.3. Rehydrate the colon section in a series of decreasing ethanol concentrations, i.e., 70%, 50%, 30% and 10% ethanol for 5 min. Repeat each step three times.

4.4. Rinse the glass slide under running water for 5 min.

4.5. Preheat antigen retrieval buffer (10 mM sodium citrate; 0.05% Tween 20, pH 6.0) to 95–98 °C and heat the glass slide in boiling antigen retrieval solution for 10 min.

NOTE: The antigen retrieval step is optional but recommended. The unmasking solution should be optimized depending on the antibody used in the experiment.

4.6. Draw a circle around the colon section using a hydrophobic pen.

NOTE: This step is optional but recommended. The hydrophobic pen prevents waste of reagents by keeping the liquid pooled in a small volume inside marked the circle.

4.7. Incubate the section in 3% water solution of hydrogen peroxidase for 10 min.

4.8. Wash in washing solution (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.05% Tween 20) for 5 min.

4.9. Incubate in blocking solution (5% normal goat serum; 50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.05% Triton X-100) for 1 h at room temperature.

NOTE: In the blocking solution, the normal serum must be from the same species as the secondary antibody. In stages where incubation is required, place the glass slide in a humidity chamber to prevent excessive evaporation.

4.10. Remove the blocking solution and add 20–50 µL of primary antibody against ERα, ERβ or GPER diluted in 1% bovine serum albumin with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Triton X-100.

NOTE: Recommended dilutions of primary antibodies are shown in **Table 2**.

[Place **Table 2** here]

4.11. Incubate with primary antibody overnight at 4 °C in darkness.

4.12. Remove the antibody solution and wash in washing solution (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.05% Tween 20) for 5 min. Repeat this step three times.

4.13. Add 20–50 µL of DyLight 650 secondary antibody diluted in 1% bovine serum albumin (containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Triton X-100). Incubate with secondary antibody conjugated with dye for 1 h at room temperature in darkness.

NOTE: The recommended dilution of the secondary antibody is shown in **Table 2**.

4.14. Remove the antibody solution and wash in washing solution (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 5 min. Repeat this step three times.

4.15. Add 2% DiOC6(3) diluted in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and incubate for 10 min at room temperature in darkness.

4.16. Remove the solution and wash in washing solution (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 5 min. Repeat this step three times.

4.17. Add a few drops of glycerol-based liquid with DAPI directly on the colon section and cover carefully with a cover slide. Incubate the colon section for least 24 h at 4 °C.

NOTE: Avoid air bubbles when covering the tissue with the cover slide.

4.18. Analyze the colon section under confocal microscope featuring 20x or 63x objectives and oil immersion using dedicated software.

NOTE: **Table 3** lists characteristics of the fluorochromes used in this study.

[Place **Table 3** here]

REPRESENTATIVE RESULTS:

Macroscopic characteristics of colons in mice with TNBS-induced Crohn's disease

Representative images of colons taken from control and TNBS-treated mice are shown in **Figure**

2. In mice with a TNBS-induced model of Crohn's disease, the length of the colon is reduced while the width of the colon is increased.

[Place **Figure 2** here]

The evaluated macroscopic parameters are given in **Table 1**. Administration of TNBS to mice leads to an increase in the total colonic macroscopic score (**Figure 3A**) and inflammation length (**Figure 3B**) relative to the control mice.

[Place **Figure 3** here]

Estrogen receptor antibody validation

Validation of the specificity of the estrogen receptor antibodies used in the study was performed using MCF-7 cells. MCF-7 cells were chosen based on previous studies wherein several independent researchers found that estrogen receptors are present at the mRNA and protein levels. As shown in **Figure 4**, the antibodies used in the study allow the detection of both nuclear estrogen receptors, i.e., ERα (**Figure 4A**) and ERβ (**Figure 4B**), as well as the membrane-bound estrogen receptor, i.e., GPER (**Figure 4C**) in MCF-7 cells. Nuclear estrogen receptors are localized in the cytoplasm and nuclei, and the signal from GPER staining is only present in the cytoplasm of MCF-7 cells.

[Place **Figure 4** here]

In addition to the positive control, a negative control was also performed, in which only the secondary antibody was used. **Figure 5** shows an image of MCF-7 cells stained only with secondary antibody conjugated with fluorochrome and glycerol-based liquid with DAPI.

[Place **Figure 5** here]

Estrogen receptor localization in the TNBS-induced murine model of Crohn's disease

A strong cytoplasmic signal of ERα was found in the colon section obtained from control mice and mice with TNBS-induced Crohn's disease (**Figure 6A**). However, it appears that only in the intestine obtained from control mice had ERα localized in the goblet cell cytoplasm. Confocal microscopy also revealed cytoplasmic localization of ERβ in the colon section of both control and TNBS-treated mice (**Figure 6B**). Similarly, cytoplasmic localization of GPER was documented in the colon section obtained from control mice and TNBS-treated mice (**Figure 6C**).

[Place **Figure 6** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Timeline for TNBS-induced murine model of Crohn's disease.

Figure 2: Representative colon obtained from the control mice (control) and TNBS-treated mice

(TNBS).

Figure 3: Total macroscopic score of the colon (A) and total colonic inflammation length (B) in control mice (control) and TNBS-treated mice (TNBS). Ten mice per group. Statistical analysis was performed using One-Way ANOVA followed by Newman-Keuls post-hoc test. Data are presented as means ± SEM; $^{***}p < 0.001$ TNBS vs. control.

Figure 4: Representative images of immunofluorescence staining of ERα (A), ERβ (B) and GPER (C) in the MCF-7 cells. Detailed description at the top of the images. Scale bars: 10 µm.

Figure 5: Representative images of immunofluorescence staining of DyLight 650 in the MCF-7 cells. Additional description is available above each image. Scale bars: 20 µm.

Figure 6: Representative images of immunofluorescence staining of ERα (A), ERβ (B) and GPER (C) in the colon section obtained from control mice (control) and TNBS-treated mice (TNBS). Additional description is available above each image. Scale bars: 50 µm; zoom scale bars: 25 µm.

Table 1: Macroscopic scoring of the intestine of mice with TNBS-induced model of Crohn's disease.

Table 2: Characteristics of antibodies.

Table 3: Characteristics of fluorochromes.

DISCUSSION:

There are numerous animal models for IBD pathophysiology examination, including genetic, immunological or spontaneous models, as well as chemically induced models[15]. Among the several types of animal models of colitis, chemically induced models such as the TNBS-induced model described in this protocol, are relatively inexpensive and easy to obtain. The TNBS-induced murine model of colitis has several clinical symptoms related to the pathological basis of CD. Animals with induced colitis are characterized by inconsistent stool formation, bloody diarrhea and loss of body weight. However, this does not mean that this model can be used to study CD etiopathogenesis exclusively. The TNBS-induced model is recommended and commonly used, for instance, for potential therapeutic screening. In the case of chemically induced colitis some critical points need to be highlighted. The TNBS has to be diluted in ethanol, which disturbs the mucosal barrier and allows TNBS to penetrate through the intestine wall and interact with high molecular weight proteins, leading to a cellular mediated immune response[2,16,17]. Both the TNBS dose and ethanol concentration should be optimized for the mouse strain and weight. Too high a TNBS dose and ethanol concentration may cause excessive mortality, which prevents further analysis. On the other hand, too low a TNBS dose and ethanol concentration may cause poor response and unnecessarily prolong the experiment.

The intestine obtained from mice with TNBS-induced colitis can be examined not only at the macroscopic level, as described in this protocol, but may also be used for biochemical and

molecular analysis. One useful approach for studying both expression and localization is an immunohistochemical technique with the use of immunofluorescence. However, some critical steps must be included in the preparation and implementation of IHC for formalin-fixed paraffin-embedded murine colon sections. The first pivotal step, i.e., preparation of the colon determines the quality of the results. The fixation time, which depends on the tissue thickness has to be optimized. Another crucial stage is dehydration, which should be performed gently by multiple incubations in increasing concentrations of ethanol. Finally, the correct positioning of the colon in the mold is essential to generate the correct cross-sections. Tissue preparation is not the only important issue in immunohistochemistry. Although antigen retrieval is an optional step, in formalin-fixed paraffin-embedded sections it appears to be necessary. During fixation with formaldehyde, methylene bridges between proteins are generated and protein crosslinking masks antigen sites[18]. There are two principal methods, based on heat- or enzymatic-induced (trypsin, pepsin or proteinase K) antigen retrieval. Heat-induced antigen retrieval, carried out in sodium citrate buffer, ethylenediaminetetraacetic acid (EDTA) buffer or Tris-EDTA buffer, is more widely used because it does not affect cell morphology. The type of antigen uptake and the conditions should be adjusted experimentally. It should be noted that sometimes the antigen retrieval method is determined by the antibody used in the experiments. Permeabilization is a condition dependent on the examined antigen, and is required especially for intracellular proteins. There are several approaches that use solvents (acetone or methanol) and harsh (Triton X-100 or NP-40) as well as mild (Tween 20 or saponin) detergents. In the present protocol two detergents, were used simultaneously, i.e., Triton X-100 and Tween 20 depending on the step. It should be emphasized that permeabilization has to be optimized depending on the antibody used. Blocking of non-specific binding sites is particularly important during immunohistochemical analysis. The blocking solutions include normal serum, bovine serum albumin or even ready-to-use blocking solutions. The present protocol recommends the use of both normal serum and bovine serum albumin. As already mentioned in the protocol, the blocking solution should contain normal serum from the same species as the secondary antibody.

Finally, IHC detection by immunofluorescence as described in this protocol can be extended to staining other proteins in protein-protein interaction studies. When looking for colocalization of selected proteins, certain conditions must be fulfilled. Fluorochromes should be selected based on excitation and emission spectra. This step is crucial to eliminate spectral overlaps and must be perform at the planning stage of the experiment. In this protocol three fluorochromes, i.e., DyLight 650 secondary antibody, DAPI and DiOC6(3) are used. As shown in **Table 3**, DyLight 650 used for estrogen receptor detection is observed as a red dye with 654 nm excitation and emission at 660–680 nm. To stain cell nuclei and the internal membrane, DyLight 650 is used along with DAPI nuclear marker and DiOC6 (3) membrane marker. DAPI is observed as a blue dye with 405 nm excitation and emission at 460–480 nm. In turn, DiOC6(3) is observed as a green dye with 485 nm excitation and emission at 538–595 nm. Staining of the next protein should be performed after step 4.14., beginning with the blocking step (see step 4.9.). For two proteins, it is recommended to use staining antibodies from different species. This approach makes it possible to exclude binding of the dye-conjugated secondary antibody to the previously stained protein.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Zhang, Y. Z., Li, Y. Y. Inflammatory bowel disease: pathogenesis. *World Journal of Gastroenterology*. **20** (1), 91 – 99 (2014).
2. Morris, G. P. et al. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology*. **96** (3), 795 – 803 (1989).
3. Okayasu, I. et al. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology*. **98** (3), 694 – 702 (1990).
4. Boirivant, M., Fuss, I. J., Chu, A., Strober, W. Oxazolone colitis: a murine model of T helper cell type 2 colitis treatable with antibodies to interleukin 4. *Journal of Experimental Medicine*. **188** (10), 1929 – 1939 (1998).
5. Morrissey, P. J., Charrier, K., Braddy, S., Liggitt, D., Watson, J. D. CD4+ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4+ T cells. *Journal of Experimental Medicine*. **178** (1), 237 – 244 (1993).
6. Kühn, R., Löhler, J., Rennick, D., Rajewsky, K., Müller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. **75** (2), 263 – 274 (1993).
7. Sundberg, J. P., Elson, C. O., Bedigian, H., Birkenmeier, E. H. Spontaneous, heritable colitis in a new substrain of C3H/HeJ mice. *Gastroenterology*. **107** (6), 1726 – 1735 (1994).
8. Harnish, D. C. et al. Beneficial effects of estrogen treatment in the HLA-B27 transgenic rat model of inflammatory bowel disease. *American Journal of Physiology Gastrointestinal and Liver Physiology*. **286** (1), 118 – 125 (2004).
9. Pierdominici, M. et al. Linking estrogen receptor β expression with inflammatory bowel disease activity. *Oncotarget*. **6** (38), 40443 – 40451 (2015).
10. Włodarczyk, M. et al. G protein-coupled receptor 30 (GPR30) expression pattern in inflammatory bowel disease patients suggests its key role in the inflammatory process. A preliminary study. *Journal of Gastrointestinal and Liver Diseases*. **26** (1), 29 – 35 (2017).
11. Mohammad, I. et al. Estrogen receptor α contributes to T cell-mediated autoimmune inflammation by promoting T cell activation and proliferation. *Science Signaling*. **11** (526), eaap9415 (2018).
12. Jacenik, D. et al. G protein-coupled estrogen receptor mediates anti-inflammatory action in Crohn's disease. *Scientific Reports*. **9** (1), 6749 (2019).
13. Prossnitz, E. R., Barton, M. The G protein-coupled estrogen receptor GPER in health and disease. *Nature Review Endocrinology*. **7** (12), 715 – 726 (2011).
14. Yaşar, P., Ayaz, G., User, S. D., Güpür, G., Muyan, M. Molecular mechanisms of estrogen-

441 estrogen receptor signaling. *Reproductive Medicine and Biology*. **16** (1), 4 – 20 (2016).
442 15. Pizarro, T. T., Arseneau, K. O., Bamias, G., Cominelli, F. Mouse models for the study of
443 Crohn's disease. *Trends in Molecular Medicine*. **9** (5), 218 – 222 (2003).
444 16. Neurath, M. F., Fuss, I., Kelsall, B. L., Stüber, E., Strober, W. Antibodies to interleukin 12
445 abrogate established experimental colitis in mice. *Journal of Experimental Medicine*. **182** (5),
446 1281 – 1290 (1995).
447 17. Ikeda, M. et al. Simvastatin attenuates trinitrobenzene sulfonic acid-induced colitis, but
448 not oxazalone-induced colitis. *Digestive Diseases and Sciences*. **53** (7), 1869 – 1875 (2007).
449 18. Thavarajah, R., Mudimbaimannar, V. K., Elizabeth, J., Rao, U. K., Ranganathan, K. Chemical
450 and physical basics of routine formaldehyde fixation. *Journal of Oral Maxillofacial Pathology*. **16**
451 (3), 400 – 405 (2012).

Figure 1

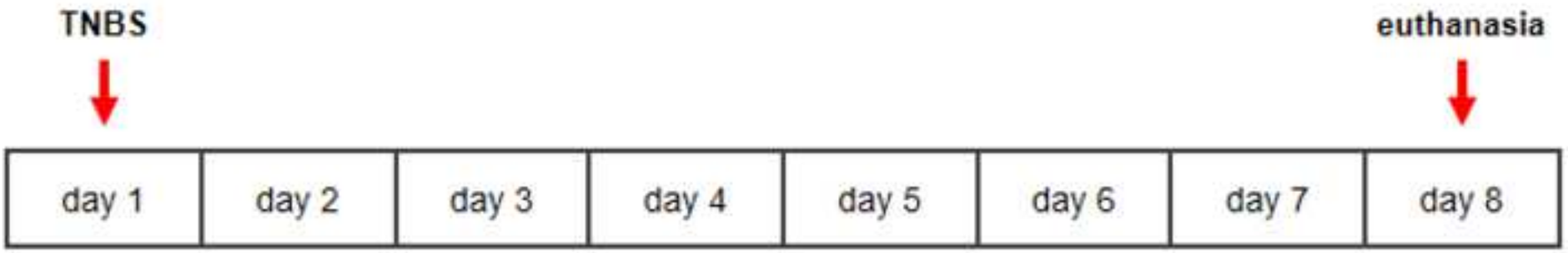

Figure 2

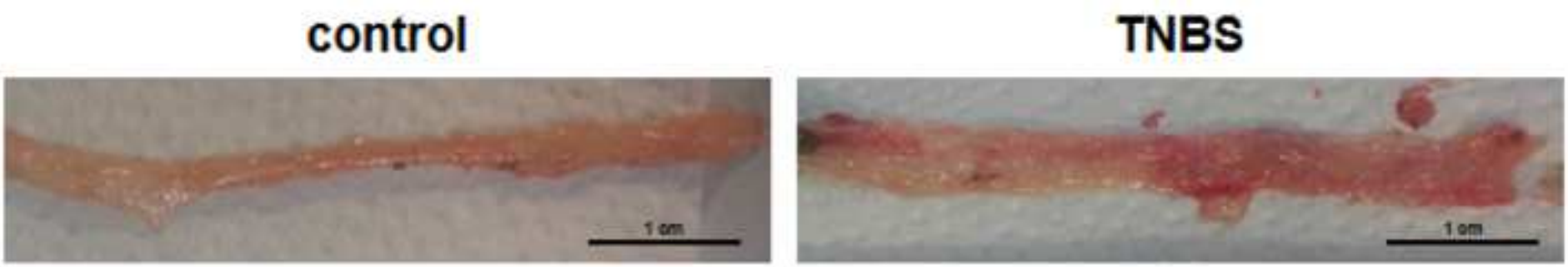

Figure 3

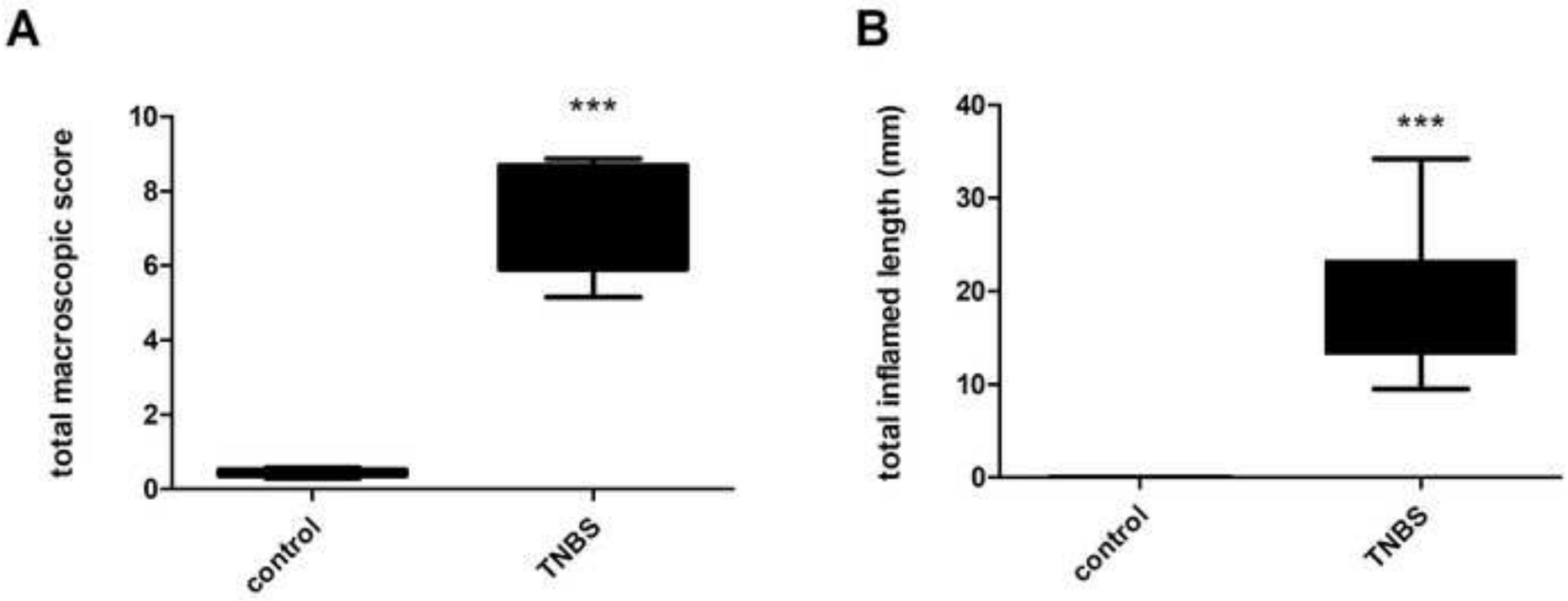

Figure 4

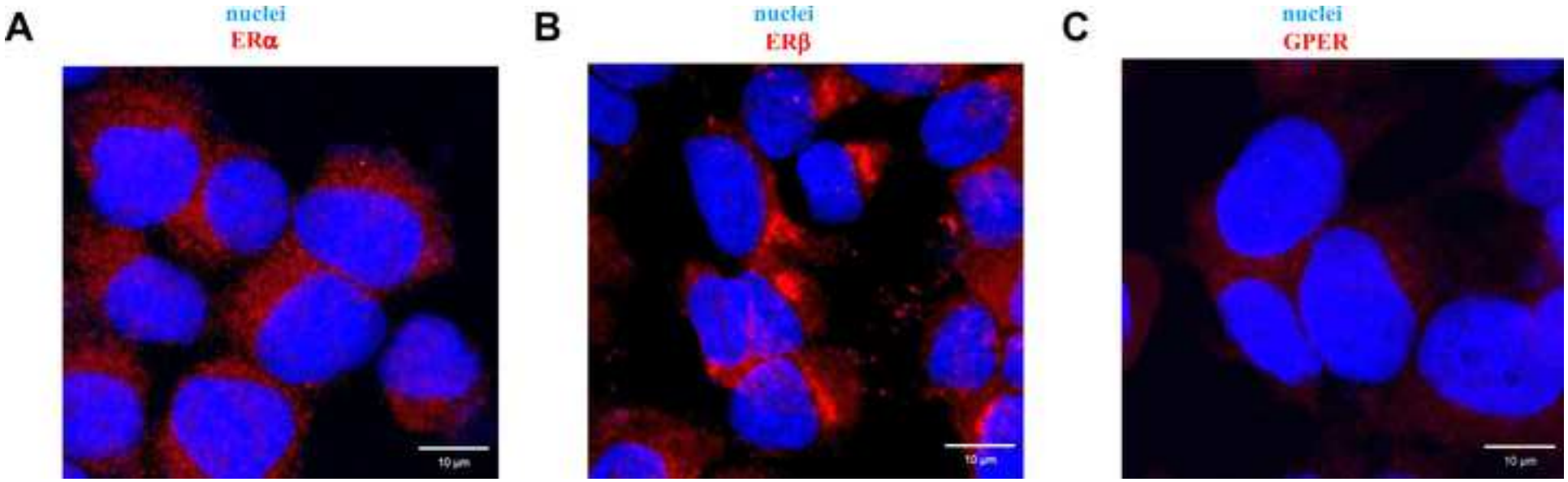

Figure 5

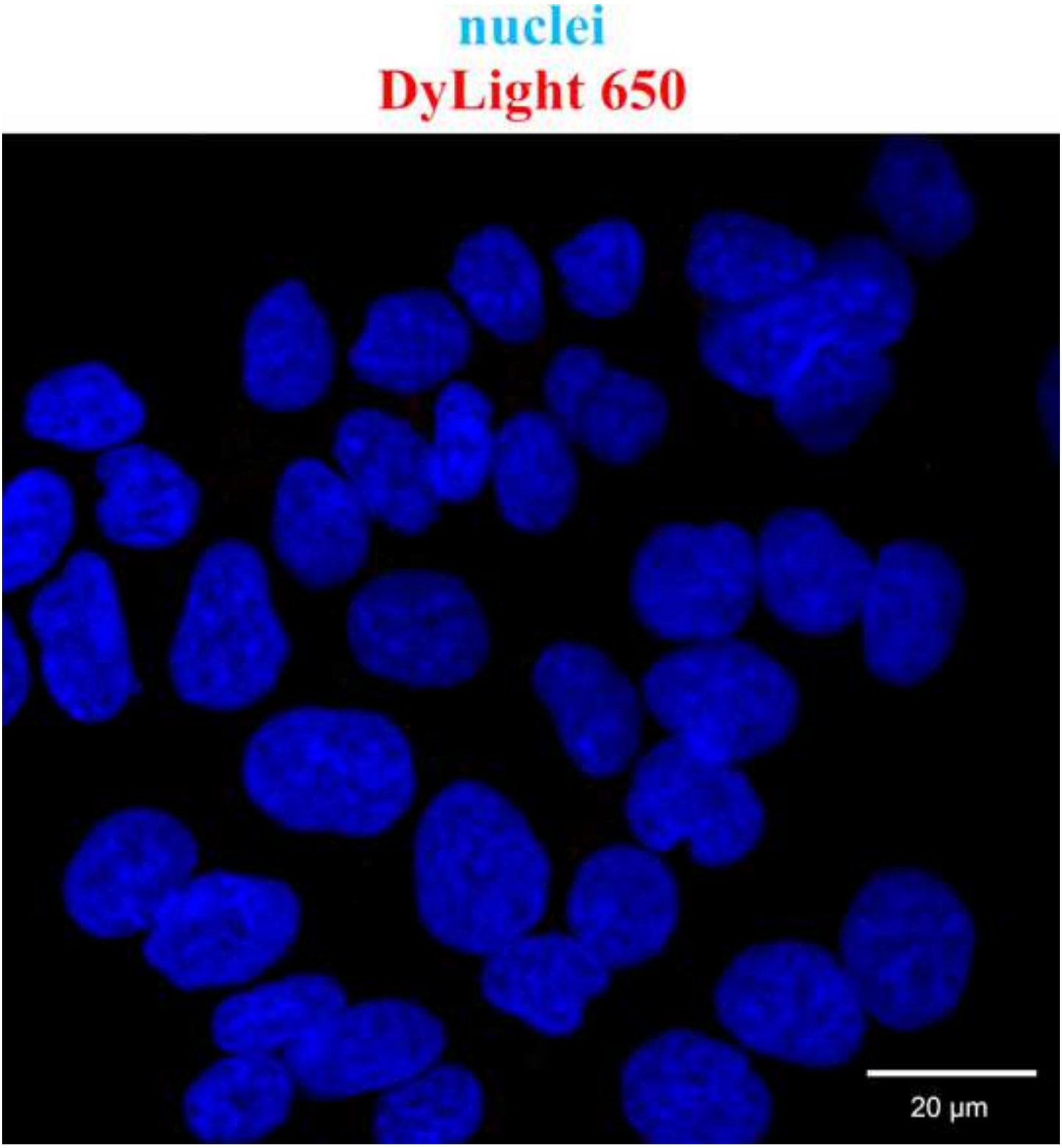

Figure 6

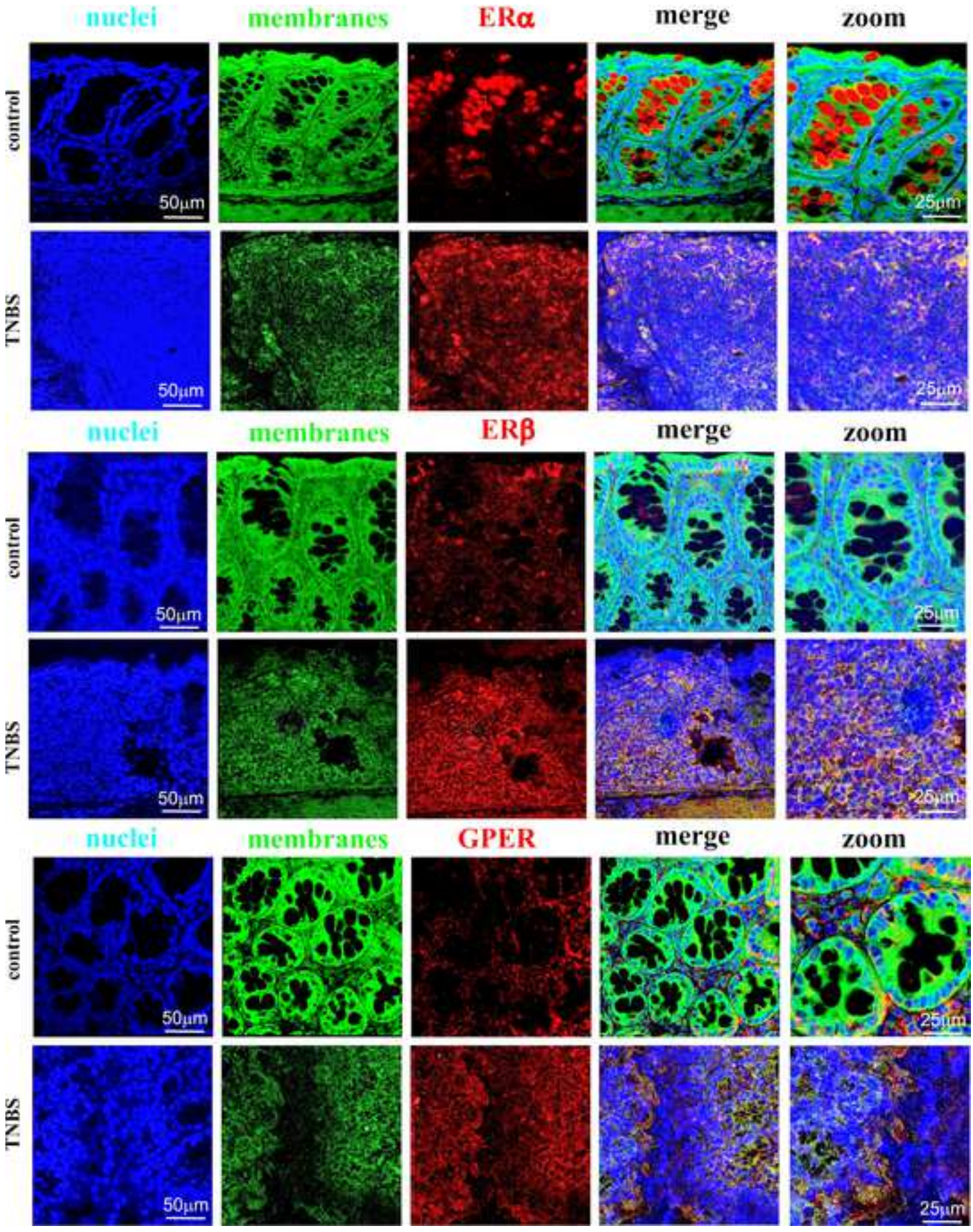

Table 1

Adhesion *	**Erythema/ hemorrhage** #	**Fecal blood** #	**Diarrhea** #	**Length of ulcer**
points (0 – 2)	points (0 – 1)	points (0 – 1)	points (0 – 1)	cm/points
0 – absent 1 – moderate 2 – present	0 – absent 1 – present	0 – absent 1 – present	0 – absent 0.5 – slight/loose stool 1 – present	0.5 cm = 0.5 point

Colon thickness	**Colon length**
mm/points	cm/points
n mm = *n* points	0 – <10% shorter than the control 1 – from 10 to 20% shorter than the control 2 – over 20% shorter than the control

Table 2

Antibody type	Antibody against	Clonality	Host species	Species reactivity	Dilution
Primary	ERα	Polyclonal	Rabbit	Human Mouse Turtle Capybara	0.111111111
	ERβ	Polyclonal	Rabbit	Human Monkey Rat Mouse Sheep Pig	
	GPER	Polyclonal	Rabbit	Human Rat Mouse	
Secondary	DyLight 650	Polyclonal	Goat	Rabbit	0.215277778

Table 3

Fluorochome type	Wavelength (nm)		Dye
	Excitation	Emission	
DAPI	405	460 – 480	Blue
DiOC6 (3)	485	538 – 595	Green
DyLight 650	654	660 – 680	Red

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Animals		
BALB/C mice	University of Lodz	NA
Equipment		
Caliper	VWR	62379-531
Cardboard block	NA	NA
Confocal microscope - TCS SP8	Leica Biosystems	NA
Fully automated rotary microtome - RM2255	Leica Biosystems	NA
Glass slide	Thermo Scientific	J1800BMNT
Heated Paraffin Embedding Module - EG1150 H	Leica Biosystems	NA
Histological box	Marfour	LN.138747
Hydrophobic pen	Sigma-Aldrich	Z377821
Laboratory balance	Radwag	WL-104-0048
LAS X software	Leica Biosystems	NA
Metal mold	Marfour	CP.5105
Sterile gauze	NA	NA
Sterile scissor	NA	NA
Sterile tweezer	NA	NA
Tissue processor - TP1020	Leica Biosystems	NA
Reagents		
2, 4, 6-trinitrobenzene sulfonic acid	Sigma-Aldrich	92822
Bovine serum albumin	Sigma-Aldrich	A3294
DiOC6 (3)	Sigma-Aldrich	318426
DyLight 650 secondary antibody	Abcam	ab96886
ERα primary antibody	Abcam	ab75635
ERβ primary antibody	Abcam	ab3576
Ethanol	Avantor Performance Materials Poland	396480111
Formaldehyde	Avantor Performance Materials Poland	432173111
GPER primary antibody	Abcam	ab39742
Hydrochloric acid	Avantor Performance Materials Poland	575283421
Hydrogen peroxidase	Avantor Performance Materials Poland	885193111
isoflurane (forane)	Baxter	1001936040
Normal goat serum	Gibco	16210064

Paraffin	Leica Biosystems	39602012
Petrie dish	Nest Scientific	705001
Phosphate buffer saline	Sigma-Aldrich	P3813
Physiological saline	Sigma-Aldrich	7982
Primocin (antibiotic)	Invitrogen	ant-pm-1
ProLong Diamond Antifage Mountant with DAPI (glycerol-based liquid with DAPI)	Invitrogen	P36971
Sodium chloride	Chempur	WE/231-598-3
Sodium citrate	Avantor Performance Materials Poland	795780429
Tris	Avantor Performance Materials Poland	853470115
Triton X-100	Sigma-Aldrich	T8787
Tween 20	Sigma-Aldrich	P9416
Xylene	Avantor Performance Materials Poland	BA0860119

First and foremost, the authors would like to thank the Managing Editor for considering our manuscript entitled "**Visualization of estrogen receptors in the colon of mice with TNBS-induced Crohn's disease by immunofluorescence**" to be published in Journal of Visualized Experiments. We thanks the Reviewers' for their valuable comments and suggestions on how to improve the manuscript. The manuscript has been rephrased and corrected accordingly and we hope that these improvements will recommends for further processing.

Responses to the Reviewers' comments:

Reviewer #1:

Manuscript Summary: After brief introduction of TNBS-induced colitis model and macroscopic evaluation of colitis, Damian Jacenik at. al emphasized on describing an immunofluorescence staining of estrogen receptors in the colon from the TNBS-insulted mice. Although the protocol is of interest to some researchers, there are many important details should be addressed in this protocol.

Major Concerns:
1. For TNBS-induced colitis model part, should add pre-sensitization step. Additionally, it would be better to use 0.1 ml 30% ethanol in 0.9% NACL as a vehicle control, instead of 0.9% NACL.

In numerous studies it was revealed that pre-sensitization step may be used to mimic the delayed type hypersensitivity reaction. In TNBS-induced murine model of Crohn's disease without pre-sensitization step we observed a significant infiltration of the immune cells in the colon, which was determined by myeloperoxidase assay, as well as some disturbances at the molecular level manifested by increased expression of several cytokines, i.e. *Il-1β*, *Il-6*, *Il-17* or *Tnf-α* in the colon of TNBS-treated mice in relation to untreated mice (reference PMID: 30179653, 30683828, 31043642, 28724693, 28333341). In our opinion, the reproducibility of the results presented in the previous and current works indicates that the experimental model used without the initial stage is proper.

In our study 0.1 mL 30% ethanol in 0.9% NaCl as a vehicle control was used. The missing information has been added to the revised version of the manuscript:

(line: 95-97) "Note: The catheter should be enter carefully approximately 3 cm into the anus. 4 mg of TNBS in 0.1 mL of 30% ethanol in 0.9% NaCl or 30% ethanol in 0.1 mL of 0.9% NaCl as a vehicle control were used."

2. The criteria of macroscopic evaluation should be cited properly, and the Table 1 should be expanded more detailly.

According to the reviewer suggestion table 1 has been improved.

3. Table of material should be included.

The material table has been added as a separate Excel file.

4. Why not use Swiss-rolling technique for colon preparation? It helps us to assess the complete colon tissues.

We agree that the Swiss-rolling technique helps in assessment of the complete intestinal or colonic sections examined. Nevertheless, in our study we used cross-section as an example

and one of the possibilities to prepare murine colon for immunohistochemical staining using immunofluorescence.

5. Please provide more clear figures, especially Figure 4. It is hard to tell the staining quality.

In the revised version improved figures have been added.

Minor Concerns:
1. Line 85, the section title should be changed, since only TNBS-induced colitis model was described in this section.

According to the reviewer suggestion, title of subsection has been changed:

(line: 84): “TNBS-induced murine model of Crohn’s disease”

2. Should describe the function of DiOC6 (3).

The following explanation in the section *Discussion* has been added to the revised version of the manuscript:

(line: 361-367) “In these protocol three fluorochomes, i.e. DyLight 650 secondary antibody, DAPI as well as DiOC6 (3) are used. According to Table 3 DyLight 650 used for estrogen receptors detection is observed as a red dye with 654 nm excitation and emission at 660–680 nm. To stain cell nuclei and internal membrane DyLight 650 is used along with DAPI nuclear marker and DiOC6 (3) membrane marker. DAPI is observed as a blue dye with 405 nm excitation and emission at 460–480 nm. In turn, DiOC6 (3) is observed as a green dye with 485 nm excitation and emission at 538–595 nm.”

Reviewer #2:

Manuscript Summary: The paper of Jacenik et al illustrates the best methods for the visualization of estrogen receptors in the colon of mice with Crohn's disease by immunofluorescence. From the methodological point of view the paper reports useful information. Additionally, the article is supported by excellent iconographic documentation.

Minor Concerns:
Just the following question may be moved: since Authors addressed their study to mice with normal mucosa and chemically induced Crohn's disease-like lesions, what was the difference in estrogen receptor pattern between the two condition and which patho-physiological implications may be extrapolated from the results of this study?

Several line of evidence suggest that estrogens play a role in the modulation of immune response and nuclear estrogen receptors, i.e. ERα, ERβ as well as membrane-bound estrogen receptor, i.e. GPER may be related to the development/progression of inflammatory bowel diseases. As we showed in our earlier work entitled "*Sex- and Age-Related Estrogen Signaling Alteration in Inflammatory Bowel Diseases: Modulatory Role of Estrogen Receptors*" (Int. J. Mol. Sci.; doi: 10.3390/ijms20133175 or PMID: 31261736) all estrogen receptors are expressed at the level of mRNA and protein in the colon of patients with Crohn's disease and ulcerative colitis. Estrogen receptors profile appears to be associated with the progression of both Crohn's disease and ulcerative colitis and expression of estrogen receptors is gender- and age-specific in patients with inflammatory bowel diseases. Our observations support hypothesis about crucial role of estrogens and estrogen receptors in the pathophysiology of colon. In these studies it has been shown that chemically-induced Crohn's disease in mice and the downstream analysis such as immunohistochemistry may be useful approach for evaluation of localization of estrogen receptors and significance of estrogen signaling in colon. Some important differences in estrogen receptors localization were highlighted in our study. It was proved that ERα expression profile is different from ERβ and GPER expression profile. It may be crucial because ERα seems to be localized mainly in the cytoplasm of goblet cells in the colon obtained from control mice. On the other hand, during uncontrolled immune reaction when immune cells are recruited to the inflamed tissues and colon architecture is completely destroyed, as in the case of colon obtained from TNBS-treated mice, the cytoplasmic localization of ERα in goblet cells was not noted. On the other hand, it was demonstrated that estrogen receptors expression profile and/or activity modulation is associated with several processes including cell proliferation, epithelial-mesenchymal transition or immune response regulation (reference PMID: 27283988, 31226730, 31043642). Detailed description of the significance of estrogen signaling in murine model of Crohn's disease is discussed in our paper entitled "*G protein-coupled estrogen receptor mediates anti-inflammatory action in Crohn's disease*" (Sci. Rep.; doi: 10.1038/s41598-019-43233-3 or PMID: 31043642). TNBS-induced murine model of Crohn's disease can be used as a *in vivo* system not only for estrogen signaling investigation but also for novel agonists/antagonists evaluation or drug screening for other targets.

Reviewer #3:

Manuscript Summary: This manuscript details a step-by-step protocol for performing immunofluorescent detection of estrogen receptors of TNBS-treated mouse colon tissue.

Major Concerns:
1. Antibody validation is key for correct interpretation of immunohistochemistry, and lack of appropriate validation is a major cause for irreproducible research. However, this aspect is completely missing from this protocol, and is not even discussed. In line with this, negative and positive controls to suport specific stainings are absent. The only control included is non-TNBS-treated mice colon tissue, which is not a control for antibody performance.

According to the reviewer suggestion the antibodies validation step was described in the section *Representative Results "Estrogen receptor antibodies validation"*:

(line: 246-253) "Validation of the specificity of estrogen receptor antibodies used in the study was performed using MCF-7 cells. MCF-7 cells were chosen based on previous reports where several independent researchers found that estrogen receptors are present at the mRNA and protein levels. As shown on figure 4, the antibodies used in the study allow the detection of both nuclear estrogen receptors, i.e. ERα (Figure 4A) and ERβ (Figure 4B) as well as membrane-bound estrogen receptor, i.e. GPER (Figure 4C) in MCF-7 cells Nuclear estrogen receptors are localized in the cytoplasm and nuclei and the signal from GPER staining is only preset in the cytoplasm of MCF-7 cells."

(line: 257-260) "In addition to the positive control, a negative control was also performed in which only the secondary antibody without primary one was used. Figure 5 demonstrated image of MCF-7 cells stained only with DyLight 650 secondary antibody and ProLong Diamond Antifade Mountant with DAPI."

In the revised version of the manuscript iconographic documentation for antibodies validation, i.e. negative and positive control staining, has been added.

Required information regarding antibodies are also missing (lot number, mono or poly-clonality, species raised in, and species of epitope raised against).

As suggested by the reviewer, a detailed description of the antibodies was added in Table 2.

Provider's webpage show that the antibodies used are raised towards either human or rat estrogen receptors, not mouse, and it is not convincingly shown that these are specific or even correctly detect mouse receptors. The non-expected staining pattern is indicative of unspecificity: A high cytoplasmic expression of ERalpha but without nuclear staining, and in tissue without clear support for ERalpha expression (literature nor mRNA evidence). Similarly, ERbeta shows only cytoplasmic staining while it is expected to be present in the nucleus of a minority of cells (the used antibody has further been shown to stain other tissues which do not have mRNA evidence of expression).

According to the provider's website both antibodies, i.e. anti-ERβ (ab3576) and anti-GPER (ab39742) are recommended to study estrogen receptor expression/localization not only in human or rat but also in mouse cells/tissues. In line, anti-ERα antibody (ab75635) was also tested by some other customers using mouse oviduct/uterus sections. All information described above are available at the provider's website:

- https://www.abcam.com/estrogen-receptor-alpha-antibody-ab75635.html
- https://www.abcam.com/estrogen-receptor-beta-antibody-ab3576.html
- https://www.abcam.com/g-protein-coupled-receptor-30-antibody-ab39742.html

There is a lot of evidence from experimental studies using human and murine tissues as well as the Human Protein Atlas indicating that estrogen receptors are present in the colon of both species at the mRNA and protein levels (reference PMID: 31261736, 31043642, 31734251, 29122010, 28338111, 31678865).

Classic estrogen receptors, i.e. ERα and ERβ, named also nuclear estrogen receptors are two first described estrogen receptors. Both nuclear estrogen receptors activated by ligands undergo conformational changes, heat shock proteins disconnection and homo- or heterodimerisation, before being translocated into the cell nucleus. Within the nucleus, they interact with the estrogen response element directly or indirectly by transcription factors leading to the regulation of target genes expression. It should be noted that “nuclear” description doesn’t mean that ERα and ERβ are present all the time in the cell nucleus but only upon activation nuclear estrogen receptors are observed in the nucleus.

These statement is confirmed not only by several teams but also by us in additional experiments where we used MCF-7 cells and antibodies against estrogen receptors (Figure 4). On the figure below nuclear localization of ERα in the MCF-7 cells is shown using 3D visualization (Figure R1).

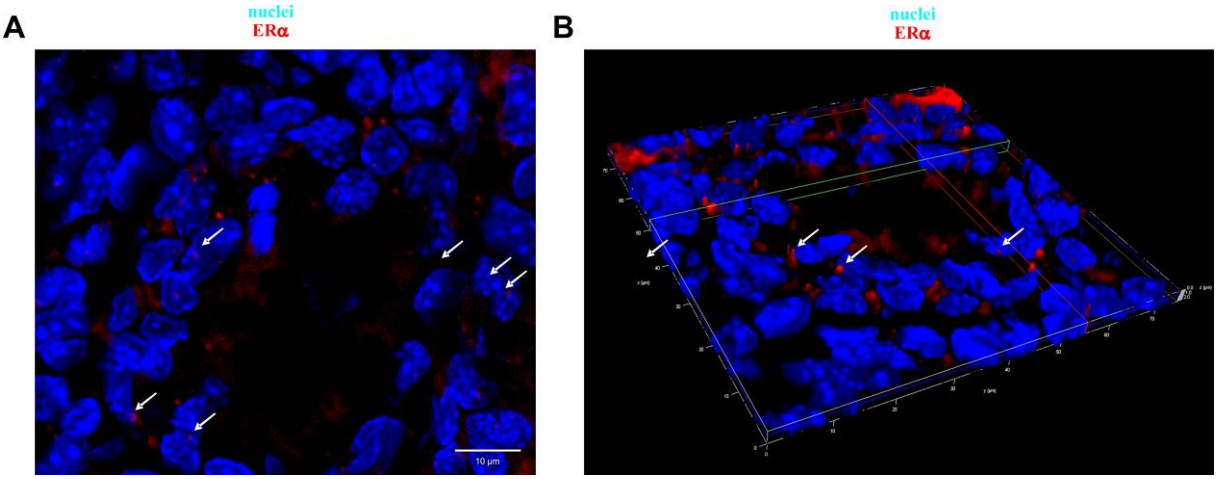

Figure R1. Representative 2D (**A**) and 3D (**B**) images of immunofluorescence staining of ERα in the MCF-7 cells – detailed description can be found at the top of the images. White arrows – nuclear localization of ERα. Scale bars, 10 µm.

2. While the tissue used for ERalpha staining are cut well (so that the length of the crypt is visible), the other tissue stainings are not, contradicting the discussion of the importance of orientation of the tissue during embedding. Further, very different areas of TNBS treated colon are shown for the ERalpha and ERbeta staining, with nearly no normal structure/crypts, whereas GPER1staining sections exhibit clear crypts. The same type of areas should be shown for direct comparison.

In the revised version of the manuscript, all cross-sections obtained from control mice and TNBS-treated mice are in the same orientation, which means that the apical side is on the top of the figure.

As suggested by the reviewer, a representative image of GPER staining representing an area similar to that of other estrogen receptors was added.

3. The control and TNBS figures in Figure 2 have completely different scales. They should be visualized with the same magnification.

According to the reviewer suggestion Figure 2 has been improved.

Minor Concerns:
**** Official gene symbols should be included (ESR1, ESR2, GPER1)***

In our paper we deal with proteins. However, according to the reviewer suggestion, in revised version of the manuscript official estrogen receptor gene symbols have been added to the section *Introduction*:

(line: 65-68) "The biological effect of estrogens is mediated by cognate receptors among which nuclear estrogen receptors (ERs), i.e. ERα (gene *ESR1*) and ERβ (gene *ESR2*) as well as G protein-coupled estrogen receptor, i.e. GPER (gene *GPER1*), characterized as a membrane-bound ER, are distinguished[13],[14]."

**** It is not clear why the colon is cut into pieces instead of using the more favored Swiss-roll technology (3.1), or why an unusually long rehydrating process is used (4.2-3). This should be justified.***

We agree that the Swiss-rolling is useful technique. Nevertheless, in our study we used cross-section only as an example and one of the possibilities to prepare murine colon for immunohistochemical staining.

For the rehydration process, we evaluated several different combination of ethanol concentration and incubation time. The presented protocol contains a procedure that allows to get the best results.

**** Table 2 should include information of catalogue number, species, mono/poly-clonality for each of the antibodies.***

According to the reviewer suggestion table 2 has been improved.

**** This is a model of Crohn's disease, but it is not Crohn's disease per se. Thus, Figure 2 figure legend, and other instances, need to be revised.***

Statement "*model of Crohn's disease*", was rearranged and changed with more adequate description.

(line: 229) "Macroscopic characteristics of colon in mice with TNBS-induced murine model of Crohn's disease"

(line: 265) " Estrogen receptors localization in TNBS-induced murine model of Crohn's disease"

(line: 279) "Figure 1. TNBS-induced murine model of Crohn's disease – timeline."

(line: 281-282) "Figure 2. Representative colon obtained from the control mice (control) and TNBS-treated mice (TNBS)."

(line: 284-285) “Figure 3. Total macroscopic score of the colon (A) and total colonic inflammation length (B) in control mice (control) and TNBS-treated mice (TNBS).”

(line: 296-298) “Figure 6. Representative images of immunofluorescence staining of ERα (A), ERβ (B) and GPER (C) in the colon section obtained from control mice (control) and TNBS-treated mice (TNBS) – detailed description at the top and left side of images.”

(line: 301-302) “Table 1. Macroscopic scoring of the intestine of mice with TNBS-induced model of Crohn’s disease.”