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## Application of Robotic Systems to Process and Embed Colonic Murine Samples for Histological Analyses --Manuscript Draft--

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**TITLE:**

Using Robotic Systems to Process and Embed Colonic Murine Samples for Histological Analyses

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**KEYWORDS:**

histopathological analysis, murine models, automation, embedding system, standardization, murine intestinal specimens

**SUMMARY:**

Lack of standardization for murine tissue processing reduces the quality of murine histopathological analysis as compared to human specimens. Here, we present a protocol to perform histopathological examination of murine inflamed and uninflamed colonic tissues to show the feasibility of robotic systems routinely used for processing and embedding human samples.

**ABSTRACT:**

The understanding of human diseases has been greatly expanded thanks to the study of animal models. Nonetheless, histopathological evaluation of experimental models needs to be as rigorous as that applied for human samples. Indeed, drawing reliable and accurate conclusions is critically influenced by the quality of tissue section preparation. Here, we describe a protocol for histopathological analysis of murine tissues that implements several automated steps during the procedure, from the initial preparation to the paraffin embedding of the murine samples. The reduction of methodological variables through rigorous protocol standardization

from automated procedures contributes to increased overall reliability of murine pathological analysis. Specifically, this protocol describes the utilization of automated processing and embedding robotic systems, routinely used for the tissue processing and paraffin embedding of human samples, to process murine specimens of intestinal inflammation. We conclude that the reliability of histopathological examination of murine tissues is significantly increased upon introduction of standardized and automated techniques.

## INTRODUCTION:

In the last decades, several experimental models have been developed to dissect the pathogenic mechanisms leading to human diseases<sup>1,2</sup>. In order to assess the severity of a disease, researchers must evaluate the effect of a treatment and study the cytological and histological architectural variations or the amount of inflammation<sup>3</sup>. To perform on those experimental models, detailed histopathological analyses are needed, often comparing murine and human samples<sup>4,5</sup>.

Additionally, human samples are commonly processed and scored by histopathology core facilities and experienced human pathologists through standardized histopathological criteria and methods. Conversely, murine tissues are usually fixed, embedded and analyzed by researchers with limited experience of histopathological protocols. The quality and reliability of histopathological examination begins with the preparation of high-quality tissue sections. Several factors critically contribute to increase or decrease the quality of the final analysis, including fixation, macroscopic sectioning, processing, paraffin embedding, and embedding of the samples<sup>6,7</sup>.

All these passages involving manipulation of the sample are subjected to manual errors, including manual embedding of the samples and, to a lesser extent, manual microtome sectioning and staining. At present, the whole process of murine tissue preparation for histological evaluation relies on protocols that vary from laboratory to laboratory and manual protocols. The goal of this study is to implement standardized automated protocols to reduce errors and variability in murine histopathological examination.

To our knowledge, we describe here the first protocols for fully automated tissue processing and embedding for the histological evaluation of murine tissues; these are routinely used in pathology units for the analyses of human specimens. As a practical example of the feasibility of the method, a murine model of intestinal inflammation has been analyzed, *i.e.*, the chronic colitis model caused by repeated administration of dextran sodium sulphate (DSS) in the drinking water<sup>8,9</sup>. This experimental setting closely resembles human inflammatory bowel diseases (IBD)<sup>10</sup> since DSS-treated animals exhibit signs of intestinal inflammation, *e.g.*, weight loss, loose stool or diarrhea, and shortening of the colon as well as fibrosis<sup>8,9,11</sup>. As observed for human IBD patients, DSS treatment generates a complex disease course. In this context, elaborate histological evaluations are required to understand the profound alteration of the tissue architecture. Thus, the implementation of the described protocols for increasing sample preparation quality might benefit researchers relying on the interpretation of histological and immunohistochemical analyses for murine experimental settings. Murine experimental models

of human diseases involving alterations of the tissue architecture, the presence of cellular tissue infiltrate or inflammation in different tissues and organs (intestine, brain, liver, skin) could use the increased quality of the sample preparation for histopathological examination.

## **PROTOCOL:**

Animal procedures were approved by the Italian Ministry of Health (Auth. 127/15, 27/13) and followed the animal care guidelines of the European Institute of Oncology IACUC (Institutional Animal Care and Use Committee)

### **1. Chronic Colitis Induction by Repetitive DSS Administration**

1.1. Separate age and sex matched mice in 2 groups (treatment DSS vs. control H<sub>2</sub>O, at least 5 mice littermates per experimental group).

1.2. Administer 2.5% DSS (40 kDa) in the drinking water for 7 days to the treatment group and water to the control group.

NOTE: This model induces a chronic transmural intestinal inflammation<sup>9</sup>.

1.3. After 7 days, stop DSS treatment and give water to both groups for 14 days. Repeat this process 3 times.

NOTE: Repetitive administration of DSS induces alterations of the colonic mucosa closely resembling human IBD, *i.e.*, fibrosis<sup>9</sup>.

1.4. Sacrifice mice according to the procedures authorized by the Institutional IACUC, *i.e.*, by CO<sub>2</sub> inhalation.

1.5. Open the mouse abdomen and peritoneum with a scalpel.

NOTE: Sterility is recommended but not strictly required.

1.6. Separate the colon from the small intestine with forceps and tweezers. Excise the colon with tweezers and forceps.

NOTE: Colon length measurement (**Figure 1A**) is a method to determine if colitis occurred in DSS-treated mice.

1.7. Rinse the colon in a Petri dish with 10 mL of cold 1x phosphate buffer saline (PBS) and gently press to remove fecal material.

### **2. Murine Tissues Fixation**

2.1. Immerse each murine colon specimen in 10 mL of 10% Neutral Buffered Formalin (NBF) at RT (room temperature).

NOTE: Sterility is recommended but not strictly required.

2.2. Fix the tissue for 18-24 h at RT.

### 3. Colon Sectioning and Tissue Preparation

3.1. Remove fixed tissues from the NBF container with small tweezers and put them in a Petri dish. Put the colon on a sectioning work plate with small tweezers.

3.2. Cut colons in fragments (0.2 cm to 0.3 cm lengths) with a sterile scalpel. This fragment length is optimal in order not to exceed the thickness of the cassette.

3.3. Pick up one colon segment with small tweezers (**Figure 2A**). Insert one colon segment into one of the plastic protruding tips of the orientated paraffin embedding cassette with small tweezers (**Figure 2B**).

3.4. Repeat the operation (3.2-3.3) with an additional 3 colon segments per cassette. Avoid inserting the segments into adjacent protruding tips, to minimize overlapping of the tissues

3.5. Tightly close the cassette by carefully pushing the four edges (**Figure 2C**). Avoid squeezing the tissue to prevent tissue damage.

3.6. Insert the grid into a plastic supporting frame. Label the supporting frame to identify the sample. Repeat for each biological sample.

### 4. Tissue Processing

4.1. Turn on the automated processor by pushing the power button (**Figure 3A, 3B**).

4.2. Warm up for 1 h to ensure paraffin wax melting. Wait until the instrument confirms the paraffin is completely melted, by observing the presence of the dedicated icon (**Figure 3C**).

4.3. Insert each oriented cassette (containing the tissue specimens) manually into the metal basket provided by the automated processor (**Figure 3D**). Place the cassettes vertically by lining them close to one to the other to optimize the basket occupancy.

4.4. Close the metal basket (**Figure 3E**).

4.5. Open the lid of the retort (**Figure 3F**). The retort is the place where the basket is inserted into the machine. Insert the basket into the dedicated housing of the processor (**Figure 3G**). Close the lid of the retort (**Figure 3H**).

177  
178 4.6. Use the touch screen on the instrument computer to define the working protocol  
179 (**Figure 3I**). Choose the sequence of solutions, timing and temperature to be implemented  
180 according to the scheme provided in **Table 1**.

181  
182 4.7. Assign the protocol to be run on the retort containing the basket by clicking on the  
183 dedicated computer icon (**Figure 3J**). Start the protocol by clicking on the **Start Button (Figure**  
184 **3L)**.

185  
186 4.8. Wait until the instrument confirms the end of the protocol, by observing the presence of  
187 the dedicated icon and by hearing the alarm tone coming from the machine.

188  
189 4.9. Open the retort lid. Remove the basket from the processor (**Figure 3M**).

## 190 **5 Tissue Embedding**

191  
192  
193 5.1. Turn on the automated embedder by pushing the power button (**Figure 4A**).

194  
195 5.2. Warm up for 1 h to ensure paraffin wax melting. Wait until the instrument confirms the  
196 paraffin is completely melted by observing the temperature of the paraffin bath indicated by  
197 the internal thermometer of the instrument.

198  
199 5.3. Manually transfer all the processed cassettes from the processor basket to the  
200 embedder rack. Each rack can contain up to 32 cassettes. (**Figure 4B, 4C**).

201  
202 5.4. Open the main embedder lid (**Figure 4C, 4D, 4E**).

203  
204 5.5. Use the touch screen on the instrument computer to signal to the robotic system that a  
205 rack is being inserted (**Figure 4F**).

206  
207 5.6. Open the inlet housing lid (**Figure 4G**). Insert the rack into the inlet housing. Each  
208 embedder can contain up to 4 racks simultaneously (**Figure 4H, 4I**). Close the inlet housing lid  
209 (**Figure 4J**).

210  
211 5.7. Use the touch screen on the instrument computer to start the embedding procedure,  
212 according to **Table 2 (Figure 4K)**.

213  
214 **NOTE:** Each rack of 32 cassettes takes 45 min to be embedded.

215  
216 5.8. Wait until the instrument confirms the end of the protocol, by observing the presence of  
217 the dedicated icon (**Figure 4L**).

218  
219 5.9. Remove the outlet rack (**Figure 4M**). Close the main embedder lid.

220

5.10. Remove the embedded blocks (containing the orientation grids) from the rack. Transfer the embedded blocks in a storage cardboard box.

## 6 Micrometer Sectioning

6.1. Turn on the cooling plate of the microtome. Set the temperature between -8 and -10 °C.

6.2. Turn on the thermostatic water bath of the microtome, containing 2 L of distilled water. Set the temperature of the water bath between 42 - 45 °C.

6.3. Place the paraffin embedded blocks on the cooling plate. Wait at least 5 min to allow the blocks to cool.

6.4. Take one block from the cooling plate and place it into the microtome block holder.

6.5. Set the microtome cut thickness to 10 µm. Trim the block by cutting it 6 times at 10 µm thickness.

6.6. Change the thickness setting of the microtome from 10 µm to 3 µm. Cut one 3 µm section for Hematoxylin and Eosin (H&E) staining.

6.7. Collect the 3 µm section with a small brush. Put the 3 µm section in the water bath, laying it carefully on the water surface to reduce tissue wrinkles. Collect the tissue section from the thermostatically controlled water bath on a single glass slide.

6.7.1. If needed, cut additional sections.

6.8. Label the glass slide by writing the sample identification.

6.9. Put the slides in a 37 °C oven for at least 10 min.

6.10. Put glass slides into racks for immediate analyses or in storage boxes.

## 7. Hematoxylin and Eosin (H&E) Staining

7.1. Switch on the automated stainer by pushing the power button. Allow the initialization of the robotic arm, by observing the change of the loading bar on the instrument monitor.

7.2. Insert the glass slides into the stainer rack, vertically inserting up to 30 slides.

7.3. Label the rack with the appropriate radio frequency identification (RFID) plastic tag. RFID tagging is a system identifying the correct staining protocol loaded into the stainer computer.

7.4. Insert the rack into the stainer. Allow the computer to automatically load and start the staining protocol according to the recognition of the RFID tag.

NOTE: The protocol for H&E staining is described in **Table 3**.

## **8. Immunohistochemical Staining**

8.1. Perform immunohistochemical and Mallory trichrome staining as previously described<sup>7</sup>.

### **REPRESENTATIVE RESULTS:**

Experimental chronic colitis induced by repeated administration of DSS in the drinking water is a murine model of intestinal inflammation closely resembling human IBD<sup>8,9</sup>. **Figure 1** describes the effects of DSS treatment, including colon shortening (**Figure 1A**), a widely-used parameter to score the presence of DSS-induced inflammation, and colonic expression of pro-inflammatory genes including *CXCL10*, *tnf* and *mcp-1* (**Figure 1B**). Infiltration of inflammatory cells was greatly enhanced by DSS treatment, showing a recruitment of the immune response in the intestinal lamina propria as analyzed by cytofluorimetry (**Figure 1C**).

**Figure 2** depicts how colonic tissues are sectioned and inserted in the oriented cassettes. These cassettes are designed to contain a grid with extruded tips, allowing the insertion of the tissue vertically and with the proper orientation, *i.e.*, with the lumen in the inner part and the wall exteriorly (**Figure 2A, 2B, 2C**). Once the cassettes are closed, the tissue orientation is preserved by the grids, contrary to what happens with conventional histological cassettes (**Figure 2B**).

The tissue processing is performed with an automated processor (**Figure 3A-3M** and **Table 1**), while the embedding procedure is performed with an automated embedder (**Figure 4A-4M** and **Table 2**). In the latter instrument, a robot collects the cassettes and dispenses the correct amount of paraffin. Finally, by using a microtome, sections are cut from each paraffin block. The slides are then prepared for further analysis.

**Figure 5** describes the main passages of the automated H&E staining (**Figure 5A-5E** and **Table 3**) and how the slides appear after H&E staining (**Figure 5F**). **Figure 6** and **7** show how the implementation of the automated processing and embedding protocols strongly increase the quality of the histopathological analyses of murine colonic specimens. The H&E stainings of samples prepared with the automated protocols derived from untreated mice (**Figure 6A**) were compared to those of DSS treated mice (**Figure 6B**). Histopathological scores were assessed by evaluating the modifications of different parameters occurring in the intestinal mucosa, including inflammatory cells infiltration, epithelial alterations and changes of the mucosal architecture as described in **Table 4**. **Figure 6C** depicts a representative H&E staining of a colonic tissue processed manually and included in traditional cassettes. In **Figure 7**, the quality of the murine tissue preparation and embedding performed through automated instruments was additionally confirmed by IHC staining of CD20 and Mallory trichrome staining<sup>8,9</sup> in colonic sections of untreated (**Figure 7A, 7C**) and DSS-treated (**Figure 7B, 7D**) mice.



**Figure 8** describes the practical relevance of this method. The same colon samples were processed and embedded either manually or through automatic methods. Each sample (either the control or DSS-treated) was cut into 2 equal parts and processed in parallel with the manual or with the automatic methods. H&E staining was then performed and a complete microscopical evaluation concerning all the pathological parameters addressing changes in the mucosal architecture, granularity, immune cell infiltrate, mucosal thickness, glandular rarefaction normally observed during intestinal inflammation, was assessed for each sample, both for the manual and automated protocol (**Figure 8A and Table 4**). The preparation of the samples with the automated protocol consistently allowed the evaluation of a higher proportion of histological parameters than the manual method. Additionally, a separate analysis of different histological parameters was performed (**Figure 8B**). The architecture and the basal infiltrate evaluation were positively affected, especially in untreated mice, by the sample preparation with the automated method.

The automated method for sample processing and embedding currently used for human histopathological analyses can be successfully applied for the analysis of murine specimens. High quality specimens are generated with the automated method that demonstrates the superiority over the manual method in the assessment of the architecture and the basal immune infiltrate of murine colonic tissues.

#### **FIGURE AND TABLE LEGENDS:**

**Figure 1: Chronic intestinal inflammation evaluation.** (A) Colon length measurement. (B) Colonic expression of pro-inflammatory genes (*cxc10*, *tnf*, *mcp-1*) in DSS-treated (black bars, n=12) and untreated mice (white bars, n=6). (C) Immunophenotyping of colonic lamina propria cells in DSS-treated (closed symbols, n=12) and untreated mice (open symbols, n=6). CD11b+Ly6G+ neutrophils, CD11b+F4/80+ macrophages (left panel), CD4+ and CD8+T cells (right panel) infiltration in controls (open symbols, n=10) and DSS-treated mice (closed symbols, n=10). Statistical significance was calculated using Wilcoxon matched-pairs signed rank t test. \*  $p \leq 0.05$  \*\*  $p \leq 0.01$ . Mean value  $\pm$  SEM are reported.

**Figure 2: Description of sample preparation.** (A) Instruments required for tissue preparation and picture of the orientated cassette, composed by an external cassette (white) and internal grid with extruded orientation tips. (B, C) Insertion of murine colons in the internal grids of the cassette before (B) and after (C) closure of the grids. In panel B is depicted the orientated cassette (left) or in a traditional cassette (right).

**Figure 3: Description of automated processing.** (A, B) automated processor. (C) Icon describing the correct paraffin wax melting temperature. (D, E) Insertion of the cassette in the metal basket (D) and its closure (E). (F, G, H) Insertion of the metal basket in the retort. (I, J, K) Selection of the processing protocol. (L) Running of the protocol. (M) Removal of the basket from the retort.

**Figure 4: Description of automated embedding.** (A) Picture of the automated embedder. (B, C) Insertion of the cassettes in the rack. (D, E) Opening (D) and closing (E) of the lid. (F) Signaling to

the machine of the presence of a rack. (G, H) Insertion of the rack into the inlet housing. (I, J) closing of the lid. (K) Start of the embedding protocol. (L) Opening of the outlet housing lid. (M) Removal of the rack form the outlet housing.

**Figure 5: Description of automated H&E stainer.** (A) Picture of the slide holder. (B, C) Insertion of the slide holder in the machine. (D) Closing of the machine. (E) Start of the staining protocol. (F) Exemplificative picture of a slide after microtome cutting and H&E staining. Right panel, H&E staining depicting the sample orientation.

**Figure 6: H&E stainings of colonic samples form untreated and DSS-treated mice.** H&E staining of untreated (A) and DSS-treated (B) samples prepared (processed, embedded, stained) with automated (A, B) or manual (C) methods. Scalebar: 100 nm.

**Figure 7: Mallory trichrome (A, B) and IHC stainings of infiltrating CD20+ cells (C, D) of untreated (A-C) and DSS treated (B, D) mice.** Mallory staining: Blue, collagen, Dark pink, nuclei, Dark red, cytoplasm. Scalebar, 100 nm.

**Figure 8: Comparison between histopathologic analyses with manual and automated protocols.** (A) Total histopathological parameters assessable in all the sections prepared either with the manual (white bars) or with the automated method (purple bars). (B) Percentage of the indicated parameters in the samples prepared with the manual (white bars) or with the automated method (purple bars) in untreated (Plain bars) or DSS-treated mice (dotted bars). Statistical significance was calculated using Wilcoxon matched-pairs signed rank t test. \*  $p < 0.05$ ; \*\*  $p < 0.005$ . Mean value  $\pm$  SEM are reported.

**Table 1: Automated tissue processing protocol**

**Table 2: Automated embedding protocol**

**Table 3: Automated staining protocol**

**Table 4: Scoring scheme for the evaluation of intestinal inflammation**

**DISCUSSION:**

We utilize different automated steps during the preparation of murine tissues for histopathologic analysis. This protocol aims at providing technical hints to increase the reproducibility and the standardization of the whole process, thus enhancing the overall quality of the final histopathological evaluation. We implemented automated instruments and methods for the preparation and embedding of tissues, routinely used in pathology core facilities for the study of human specimens.

To demonstrate the potential applicability of this method, we chose a chronic murine experimental setting of intestinal inflammation, called DSS-induced chronic colitis model. This setting resembles the complex disease course observed in IBD patients, requiring elaborate

histological evaluation to investigate the profound alterations of the tissue architecture occurring upon chronic intestinal inflammation. Altogether, the histological evaluation of these processes might greatly benefit from increased sample preparation quality. To note, this protocol can be applied to other murine tissues (*i.e.*, spleens, lymph nodes, liver, brain), with the only difference being that the oriented cassette is not needed for tissues without a lumen.

The most important observation was that the decrease of manual errors, (*i.e.*, the orientation of the sample) given the use of cassettes with grids and the elimination of cassette re-opening for the embedding (a step commonly performed during manual protocols), strongly enhanced the overall reproducibility of the analysis. With the protocol described here, the sample is manipulated only at the beginning of the preparation, when the experimenter inserts the tissue in the orientated cassettes. Once closed, the cassettes are never re-opened, thus ensuring the maintenance of the correct orientation and reducing manual errors<sup>3,11</sup>. The standardization of these two critical steps enhanced the whole quality of the subsequent analyses and decreased the number of lost or not assessable samples, problems both linked to the re-opening of the cassette or to the wrong orientation of the sample<sup>3,11</sup>.

We also succeeded in evaluating a complex biological event such as fibrosis (**Figure 7**), that is very often underestimated in murine models of experimental intestinal inflammation, by the implementation of the automated protocol. During the set-up of the protocol, we strictly standardized technical details such as the fixation time, which we realized must not exceed 24 h to avoid tissue alterations. By doing so, we could preserve the quality of subsequent immunohistochemical analyses.

The automated method improved the evaluation of those parameters associated to the alteration of the tissue architecture, especially in untreated mice. Indeed, the correct comparison of a pathological tissue with a healthy counterpart is critically important for the final assessment of the experimental model<sup>3</sup>.

Concerning the tissue processing, we tested different protocols to be run in the automated processor available in the laboratory. The protocol described here gave sound and highly consistent results. We also implemented a time length for the protocol. Since murine samples were comparable to human small biopsic specimens in size, a shorter protocol was sufficient to process murine (intestinal, in this case) tissues. Finally, the whole automated procedure reduced the total experimental time. From the beginning of the processing to the microtome cutting and slices preparation, we calculated that the total time required is 5 h. On the contrary, depending on the technical ability of the operator, the completion of the same protocols manually can require more than double the amount of time, especially during the processing and the embedding. One limit of the technique is that it requires automated processors and embedder to be performed to be in the laboratory.

In conclusion, we believe that the implementation of these automated protocols could greatly ameliorate the work of translational researchers dealing with murine experimental models of human diseases.

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**DISCLOSURES:**

The authors have nothing to disclose.

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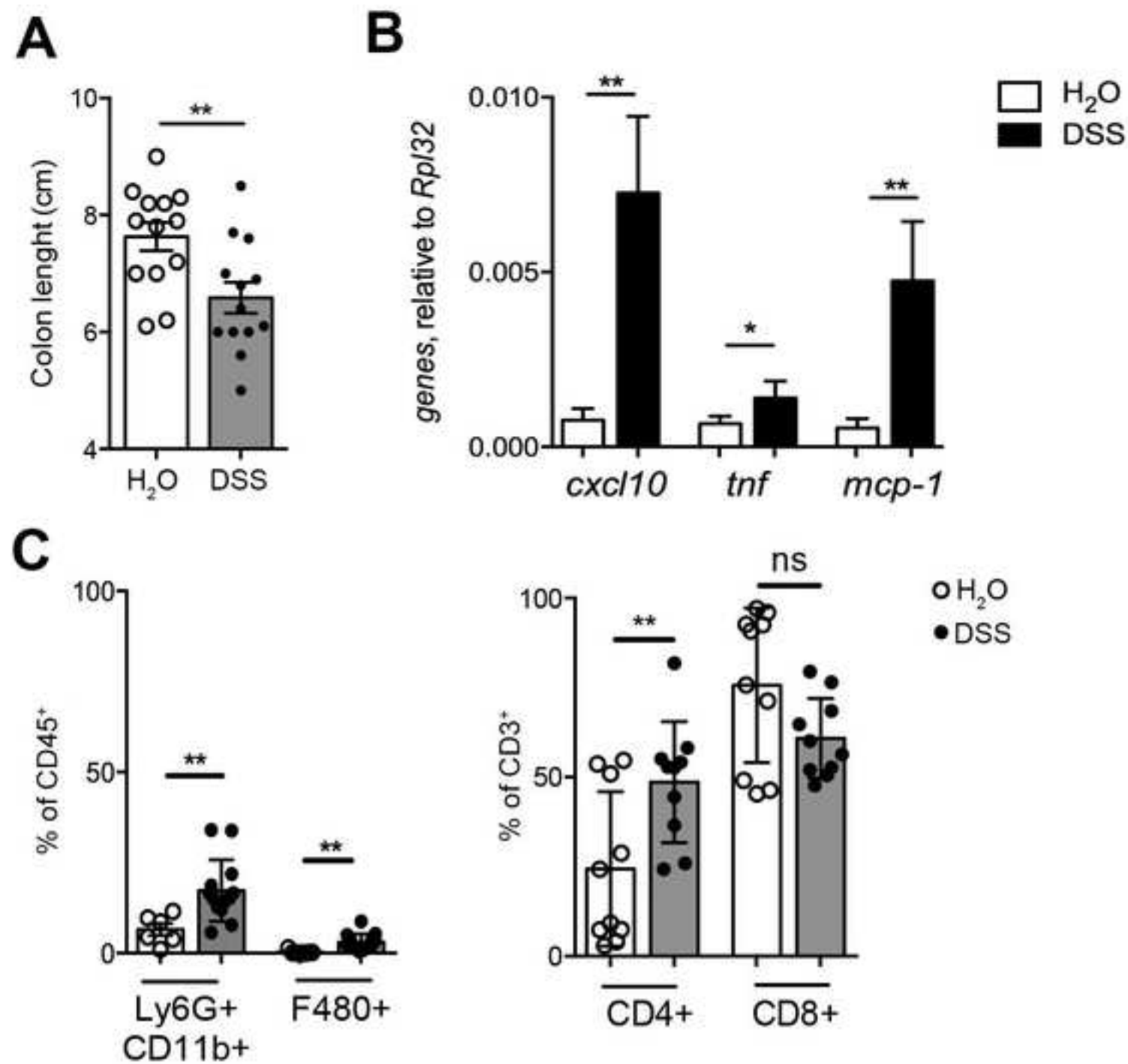
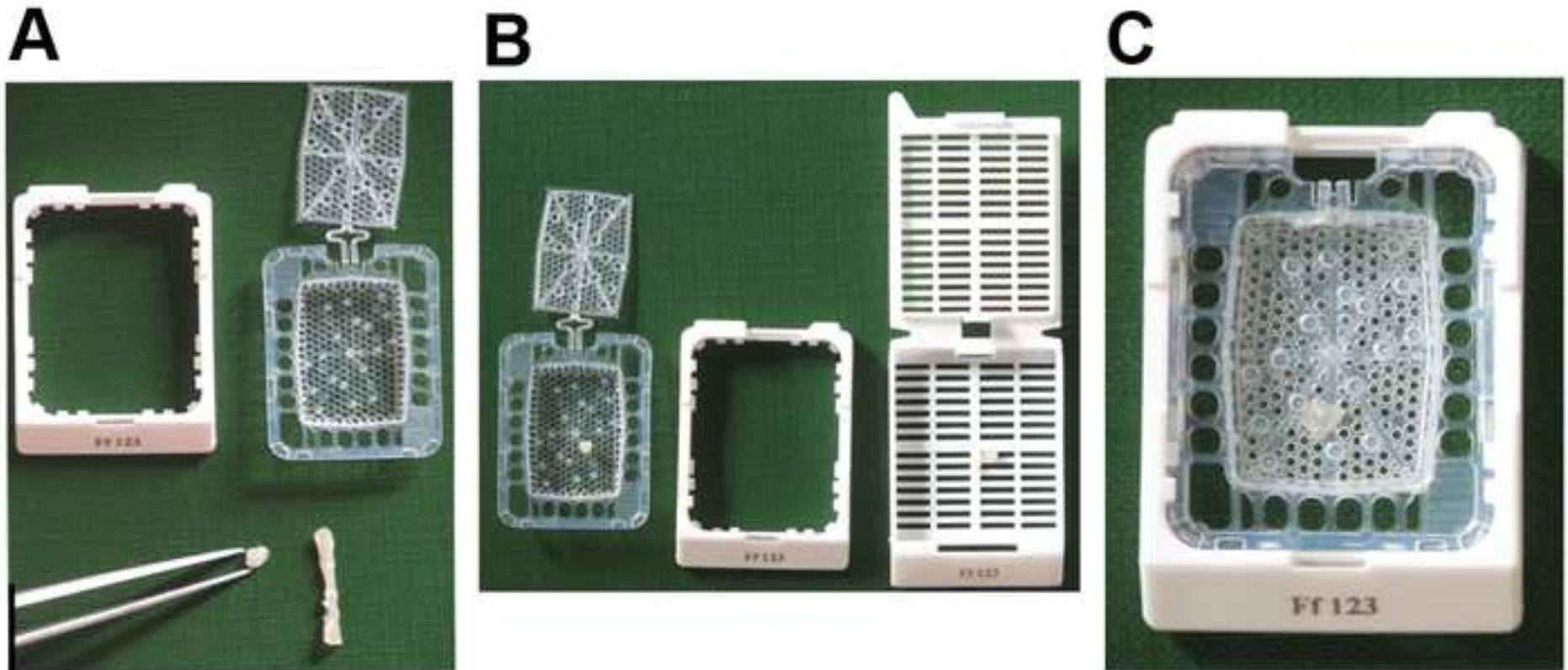


Figure 1



**Figure 2**



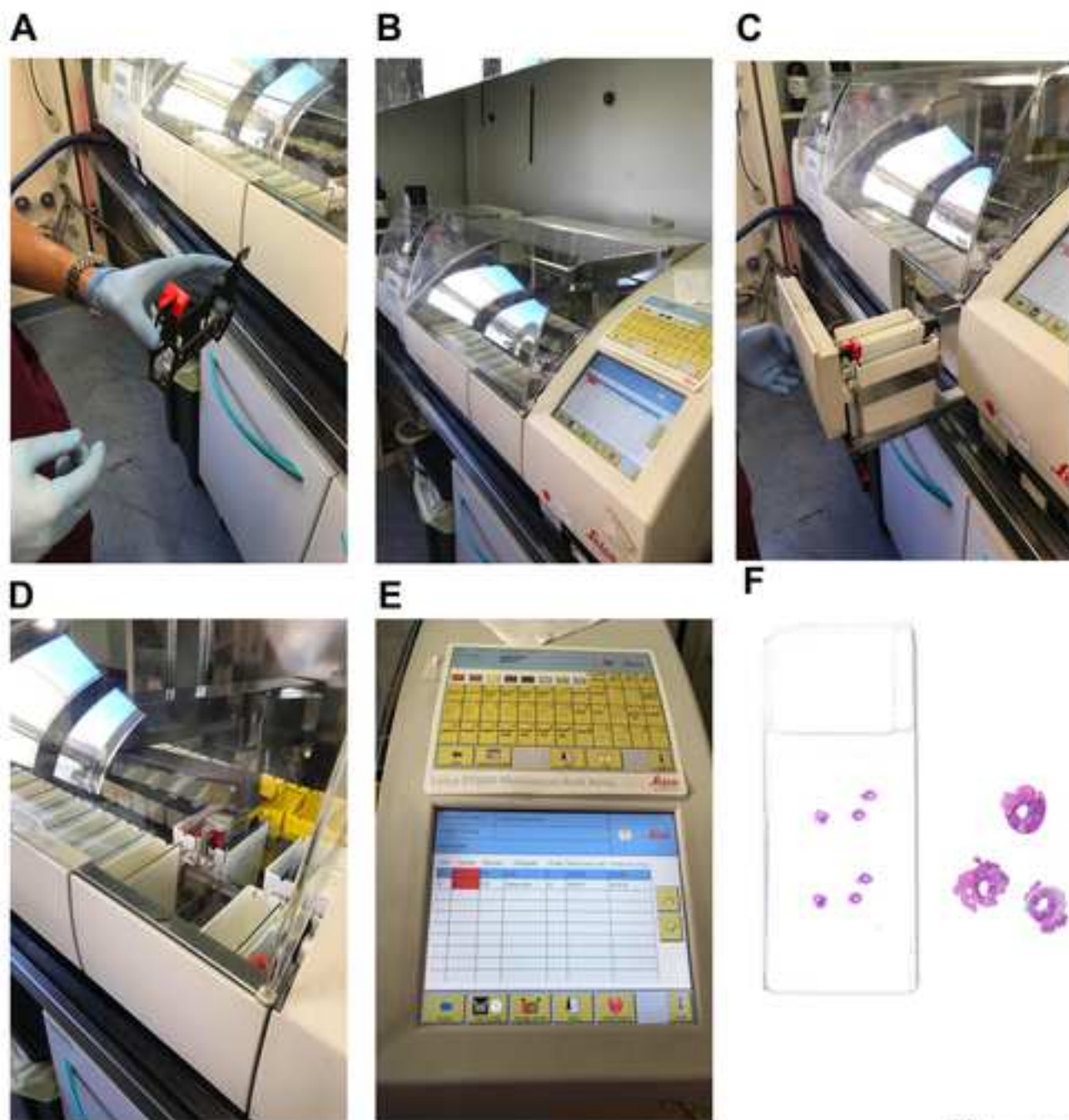


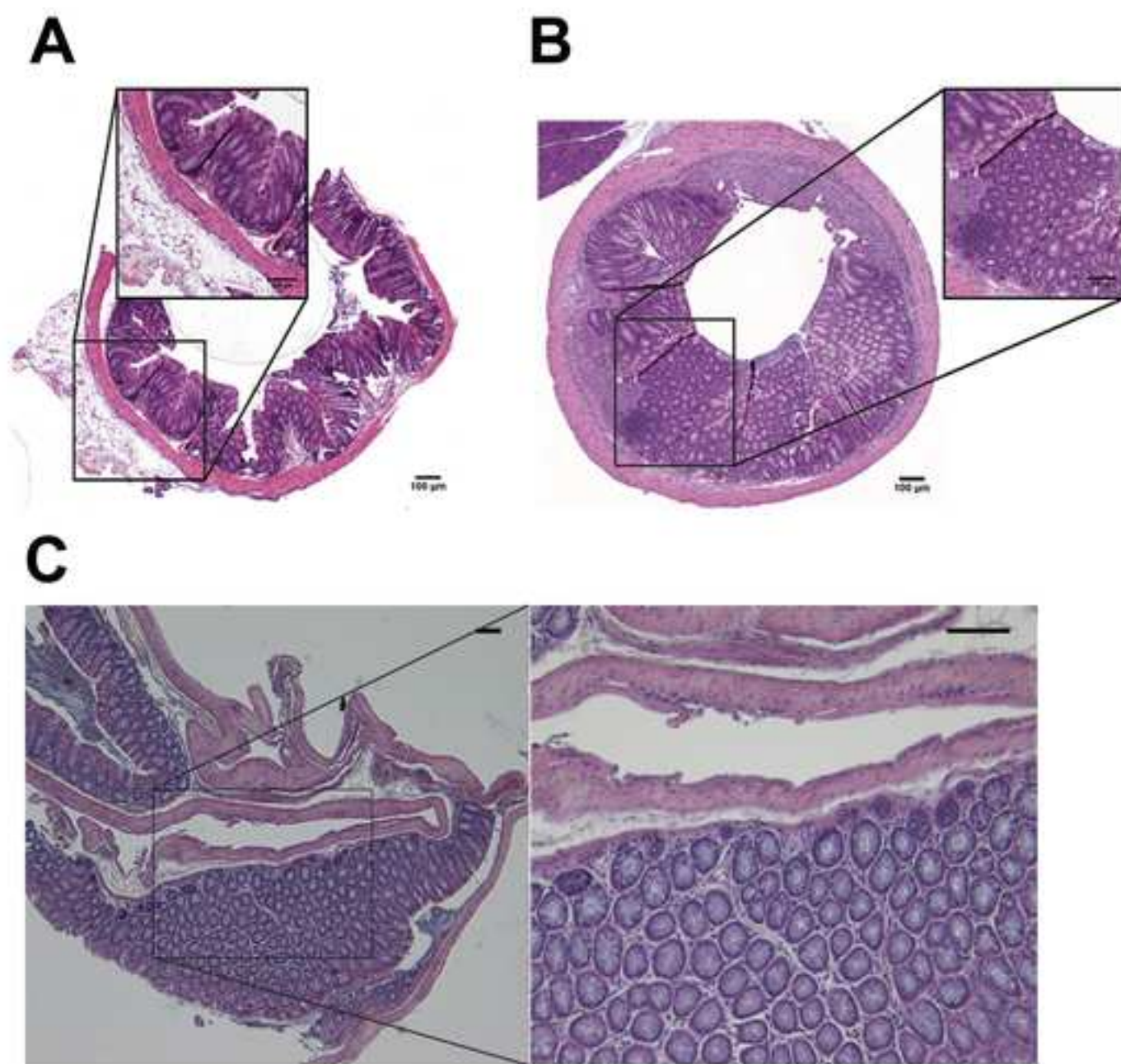
Figure 3



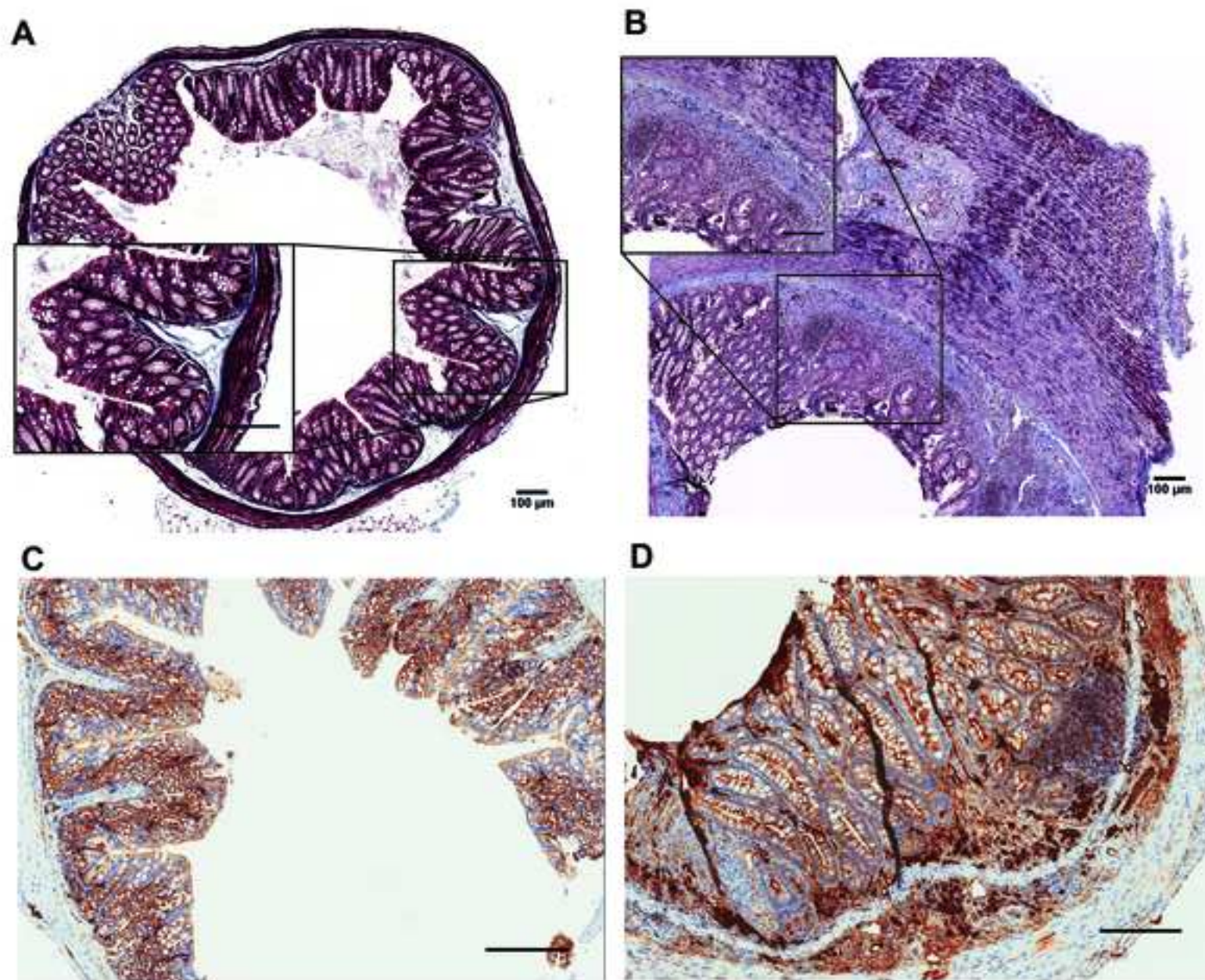
Figure 4

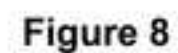


**Figure 5**

**Figure 6**



**Figure 7**



**Figure 8**

Reagent	time (min)	temperature (°C)	pressure
NBF	1	RT	ambient
Ethanol 95%	1	RT	ambient
Ethanol 95%	1	RT	ambient
Ethanol 95%	1	RT	ambient
Absolute Ethanol	1	45	ambient
Absolute Ethanol	11	45	ambient
Absolute Ethanol	30	RT	ambient
Xylene	1	RT	ambient
Xylene	1	45	ambient
Xylene	28	45	ambient
Paraffin wax	5	65	vacuum

**Action perfomed by the robot for each cassette**

- Remove one cassette from the rack
- Identify the cassette
- Pre-heat the mold
- Place the cassette on the pre-heated mold
- Dispense the amount of paraffin for the cassette
- Cool down the mold
- Allow the paraffin to solidify
- Remove the solidified paraffin block from the mold
- Present the block to quality sensors
- Place the paraffin block in the output door

Category	Criterion	Definition
Inflammatory cell infiltrate	<u>Severity</u> (leukocyte density of lamina propria area infiltrated in evaluated hpf)	No infiltrate
		Minimal acute (<10%)
		Mild chronic (10-25%, scattered neutrophils)
		Moderate chronic (26-50%)
		Marked (>51%, dense infiltrate)
Epithelial changes	<u>Extent</u> (expansion of leukocyte infiltration)	Mucosal
		Mucosal and submucosal
	<u>Hyperplasia</u> (increase in epithelial cell numbers in longitudinal crypts, visible as crypt elongation)	No hyperplasia
		Minimal (<25%)
		Mild (26-35%)
		Moderate (36-50%, mitoses in the upper third of the crypt epithelium)
		Marked (>51%, mitoses in crypt epithelium distant from crypt base)
	<u>Goblet cell loss</u> (reduction of goblet cell numbers relative to baseline goblet cell numbers per crypt)	No loss
		Minimal (<25%)
		Mild (26-35%)
Mucosal architecture		Moderate (36-50%)
		Marked (>51%)
	<u>Ulceration</u> (epithelial defect reaching beyond muscoularis mucosae)	No ulcers
		Ulcers

	<u>Granulation tissue</u> (connective tissue repair with new capillaries, surrounded by infiltrating cells, hypertrophied areas)	No granulation tissue
		Granulation tissue
	<u>Mucosal thickness and crypt depth</u>	No thickening
		Thickening
	<u>Glandular rarefaction</u>	No rarefaction
		Rarefaction
	<u>Dysplasia</u>	No dysplasia
		Dysplasia
		<b>MAX SCORE</b>



Score value
0
0.25
0.5
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6

Action performed by the st Reagent		time (s)
Essicate		180
Essicate		180
Deparaffinize	Xylene	120
Deparaffinize	Xylene	120
Hydrate	Ethanol 96%	120
Hydrate	Ethanol 96%	120
Wash	Distilled wtaer	240
Stain Hematoxylin	Carazzi's Hematoxylin	540
Rinse	Tap Water	360
Stain Eosin	Eosin Y 1% aqueos solution	60
Rinse	Tap Water	120
Dehydrate	Ethanol 96%	20
Dehydrate	Ethanol 96%	20
Dehydrate	Absolute Ethanol	15
Dehydrate	Absolute Ethanol	15
Clear	Xylene	30
Clear	Xylene	30

**temperature (°C)**

---

60

60

RT

RT

RT

RT

RT

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RT

<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
Absolute Ethanol anhydrous	Carlo Erba	414605
Absolute ETOH	Honeywell	02860-1L
Aluminium Potassium Sulfate	SIGMA	A6435
Aniline Blue	SIGMA	415049
carbol Fuchsin	SIGMA	C4165
CD11b (clone M1/70)	TONBO biosciences	35-0112-U100
CD20 IHC (clone SA275A11)	Biolegend	150403
CD3 (17A2)	TONBO biosciences	35-0032-U100
CD4 (GK1.5)	BD Biosciences	552051
CD45.2 (clone 104)	BioLegend	109837
CD8 (53-6.7)	BD Biosciences	553031
Citrate Buffer pH 6 10X	SIGMA	C9999
Dab	Vector Laboratories	SK-4100
DPBS 1X	Microgem	L0615-500
DSS	TdB Consultancy	DB001
EDTA	SIGMA	E9884
EnVision Flex Peroxidase-Blocking Reagent	DAKO	
EnVision Flex Substrate	DAKO	
EnVision Flex/HRP	DAKO	
EnVision Flex+ Rat Linker	DAKO	
Eosin	VWR	1.09844
F4/80 (clone BM8)	BioLegend	123108
Formalin	PanReac	2,529,311,215
glacial acetic acid	SIGMA	71251
Goat-anti-Rat-HRP	Agilent DAKO	P0448
Haematoxylin	DIAPATH	C0303
LEICA Rotary microtome (RM2255)	Leica	RM2255
Ly6g (clone 1A8)	BD Biosciences	551459

Mercury II Oxide	SIGMA	203793
Omnis Clearify Clearing Agent	DAKO	CACLEGAL
Omnis EnVision Flex TRS	DAKO	GV80011-2
Orange G	SIGMA	O3756
Paraffin	Sakura	7052
Peloris	LEICA	
Percoll	SIGMA	P4937
RPMI 1640 without L-Glutamine	Microgem	L0501-500
STS020	Leica	
Tissue-Teck Paraform Sectionable Cassette	SAKURA	7022
Tissue-Tek Automated paraffin embedder	Sakura	
Xylene	J.T.Baker	8080.1000

**Comments/Description**

reagent

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Author(s):

FEDERICA FACCIOTTI

\* ANALYSIS OF MURINE INTESTINAL PAPILLES

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## Point by point reply to the editor

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

*Done.*

2. Please address all the specific comments marked in the manuscript.

*Done. In order to better clarify the issues raised by the editor we added two references (number 8, 9). 3 novel figures have been added (Figs 3,4,5), 4 tables (Tables 1-4), a panel has been added to figure 1 concerning qPCR results (Fig1B), a comparison with manual and automated method has been added in figure 6 and was already present in ex Figure 5 (now Figure 8).*

*We expanded the protocol when needed, we clarified the passages, added pictures to support the text description or referred in the text to the figure as suggested by the Editor.*

*In a couple of comments the Editor questioned the appropriateness of the DSS-chronic administration to induce intestinal inflammation. This setting is widely used in the gastroenterological field to study pathological features linked to intestinal inflammation, resembling human IBD. Being IBD a chronic disease, some clinical manifestations (i.e, fibrosis) are present only upon repetitive administration of DSS, thus causing chronic inflammation.*

*Moreover, we do agree that murine experimental settings are never completely adherent to human pathologies. In this context, it is widely accepted to define them "models", even if we are aware that an experimental setting can never perfectly model a human disease. To support this practical reason we added two references that address this issue (refs 8,9)*

3. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

*Added in lines 76-77*

b) The rationale behind the development and/or use of this technique

*Present in lines 63-70 and added in lines 87-90*

c) The advantages over alternative techniques with applicable references to previous studies

*To our knowledge there are no alternative techniques beside the manual processing and embedding. Lines 79-81*

d) A description of the context of the technique in the wider body of literature

*Beside the everyday practical experience of the requirement of protocols to increase the quality of histopathological examination also for murine experiments, very few articles addressed this topic. These papers have been cited as ref 1-3*

e) Information to help readers to determine whether the method is appropriate for their application

*Added in lines 89-7*

4. For the protocol section, please include every single detail of how the experiment is

being performed. Please use imperative tense throughout as if directing someone how to perform the experiment. Please use complete sentences.

*We thoroughly corrected all the protocols.*

5. If there are any graphical user interface, button clicks, knob turns etc for the robotic system described here. We need action steps.

*We corrected the protocols. Whenever it was not possible to perform actions, being some of the actions performed by an automated instrument, we added specific Tables (Tables 1-3) and added the pictures in Figures 3-5*

6. After all the changes, please ensure that the highlight is no more than 2.75 pages including headings and spacings.

*Done.*

7. Figures: Please upload all figures individually to your editorial manager account.

*It will be done.*

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*We changed all the panels containing previously published figures or pictures and none of the figures in this current manuscript have been ever published before, therefore it is not needed anymore the copyright permission.*