

1            **Role of FBXW2 in explant culture of bovine periosteum-derived cells**

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10

## 1 **Abstract**

2           Osteoporosis and bone fracture decrease quality of life. Bone regeneration is a  
3 notable technique for osteoporosis treatment. A previous study reported that F-box and  
4 WD-40 domain-containing protein 2 (FBXW2) and osteocalcin have the same shape in  
5 the periosteum after 5 weeks. However, the osteoblastic functions of FBXW2 are not  
6 clear. In this study, double fluorescent immunostaining revealed a small amount of  
7 osteocalcin in the area of FBXW2 aggregation at 1 week, periosteal cells, and osteocalcin  
8 pushed toward the edge of periosteum, and, apart from FBXW2 tubes at 2 weeks,  
9 multilayered periosteum-derived cells at 3 weeks and sticking of osteocalcin in the  
10 periosteum with cells at 4 weeks. At 5 weeks, FBXW2 disappeared at the root of  
11 periosteum-derived cells, while osteocalcin and cells remained. Based on these results, it  
12 is hypothesized that FBXW2 maintains tissue shapes and prevents escape of inner  
13 periosteal cells, and the disappearance of FBXW2 causes migration of periosteum-  
14 derived cells out of the periosteum along with osteocalcin. Furthermore, FBXW2 may  
15 play a role in dynamic tissue remodeling and bone formation.

16

## 17 **Introduction**

18           Osteoporosis and bone fracture decrease quality of life. Bisphosphonates used

1 for the treatment of osteoporosis are associated with the risk of osteonecrosis of the jaw  
2 [1]. Besides bisphosphonates, bone regeneration is a notable treatment for osteoporosis.  
3 Many studies have investigated the role of the cambium layer of the periosteum [2–5];  
4 however, specific proteins in the periosteum that aid in bone formation are still unknown.  
5 Periosteal stem cells are also important for bone regeneration [6]. Bovine periosteum-  
6 derived cells are used for bone regeneration, and these cells can form multilayered cell  
7 sheets without scaffolds on tissue culture dishes [7]. To determine the mechanism of  
8 multilayered cell sheet formation, the supernatant and periosteum were studied [8,9].  
9 Akiyama investigated the supernatant of bovine periosteum-derived cells using mass  
10 spectrometry and immunohistochemistry [9] and found that F-box and WD-40 domain-  
11 containing protein 2 (FBXW2) is expressed in the periosteum [10]. FBXW2 is one of the  
12 F-box proteins involved in the ubiquitin-proteasome system [11]. Among the 69 known  
13 F-box proteins, only four—FBXW7, SKP2,  $\beta$ -TRCP1, and  $\beta$ -TRCP2—are well studied,  
14 whereas the functions of the remaining 65 members are still unknown [12]. Thus, the  
15 function of FBXW2 is also unknown. In 2018, Akiyama reported that FBXW2 and  
16 osteocalcin form tubes of the same shape in the periosteum after 5 weeks in culture [13],  
17 but the relationship between these two proteins is not clear. The osteoblastic function of  
18 FBXW2 is also unknown. In this study, periosteum and periosteum-derived cells were

1 observed for up to 5 weeks using double fluorescent immunostaining to determine the  
2 effects of FBXW2 on the osteoblastic character of periosteum-derived cells.

3

## 4 **Materials and methods**

### 5 **Preparation of periosteum**

6 All protocols were approved by the Animal Research Committee of Osaka  
7 Dental University (approval number 20–02006) and complied with fundamental  
8 guidelines for proper conduct of animal experiments and related activities in academic  
9 research institutions under the jurisdiction of the Ministry of Education, Culture, Sports,  
10 Science and Technology (The Ministry of Education, Culture, Sports, Science and  
11 Technology directive 2006, Notice No. 71). The periosteum was separated from the  
12 bovine leg (Kobe Chuo Chikusan, Kobe, Japan) as described previously [7]. Bone  
13 sections were removed, fixed with 4% paraformaldehyde (PFA), and cast into paraffin  
14 blocks. The periosteum was cultured in 100 mm dishes in Medium 199 supplemented  
15 with 10% fetal bovine serum, 100 units penicillin, 100 µg streptomycin/mL solution  
16 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 5 mg/mL ascorbic acid for up  
17 to 5 weeks. The medium was changed once a week. The periosteum was fixed every week  
18 and prepared for sections. Figure 1 shows the schema of this study.

1 **Fig 1. Schema of this study.**

## 2 **Fluorescent immunostaining and immunohistochemistry**

3 All paraffin sections were pre-treated with ready-to-use Proteinase K (Dako  
4 Cytomation, Glostrup, Denmark) for 10 min. Primary antibodies used were mouse  
5 monoclonal antibodies for osteocalcin (Santa Cruz Biotechnology, Inc., No. sc-376835),  
6 a mouse monoclonal antibody for bovine osteocalcin (code no. M042, clone no. OCG2;  
7 Takara Bio Inc., Shiga, Japan), and goat polyclonal antibody for FBXW2 (Invitrogen,  
8 #PA5-18189). The secondary antibodies used were Alexa Fluor™ 488 goat anti-mouse  
9 (Invitrogen, #A11029), mouse anti-goat IgG-CFL 594 (Santa Cruz Biotechnology, Inc.,  
10 No.sc516243), and N-Histofine Simple Stain AP (multi) (#414261, Nichirei Biosciences  
11 Inc., Tokyo, Japan). Alkaline phosphatase-tagged antibody was visualized with  
12 PermaRed/AP (K049, Diagnostic BioSystems, CA, USA). Incubation with mouse  
13 monoclonal antibodies for osteocalcin (diluted 1:100) was performed overnight at 4 °C,  
14 and that with monoclonal antibody for bovine osteocalcin (diluted 1:500) and FBXW2  
15 antibody (diluted 1:100) was performed for 4 h at room temperature. Cell nuclei were  
16 stained with hematoxylin or DAPI. For negative controls, the antibody for receptor  
17 activator of NF-κB ligand (RANKL) (Santa Cruz Biotechnology, Inc., No. sc-377079)  
18 and normal goat serum (Fuji film Wako Pure Chemical Industries, Ltd. 143-06561) were

1 used. Images were photographed with a fluorescence microscope (Keyence Japan, Osaka,  
2 Japan, BZ-9000).

3

## 4 **Results**

5 Figure 2 shows the bone and cambium layer. Consistent with a previous study  
6 [13], FBXW2 (red) is expressed in the bone and cambium layer (Fig 2(a)). Figure 2(b)  
7 shows negative control of the anti-FBXW2 goat antibody (red); only cell nuclei (blue)  
8 are stained. Figure 2(c) shows osteocalcin expression using a mouse monoclonal antibody  
9 for osteocalcin (No. sc-376835). As shown in Fig 2(d), RANKL is not expressed. An  
10 alkaline phosphatase labeled secondary antibody was used in Fig 2(c)–(d), and  
11 fluorescent labeled secondary antibody was used in Fig 2(e)–(f). In Fig 2(c)–(e), the same  
12 primary antibody for osteocalcin was used. Figure 2(e) reveals that osteocalcin is  
13 expressed in bone (green), but not in the cambium layer (cell nuclei stained blue). In Fig  
14 2(d) and 2(f), the same primary antibody for RANKL was used. Figure 3 shows double  
15 fluorescent immunostaining of the periosteum at day 0. In Fig 3, FBXW2 is expressed in  
16 blood vessels, while osteocalcin is not. Although osteocalcin reacts with blood cells, the  
17 reaction may be non-specific.

18 **Fig 2. Fluorescent immunostaining and immunohistochemistry of bone.** Scale bar:

1 100µm. (a) FBXW2: red, (b) negative control of (a), (c) osteocalcin, (d) RANKL: negative  
2 control of (c), (e) osteocalcin: green, (f) RANKL: negative control of (e)

3 **Fig 3. Double fluorescent immunostaining of periosteum at day 0.**

4 Osteocalcin: green, FBXW2: red, DAPI: blue Scale bar: 100µm.

5 Monoclonal antibody for bovine osteocalcin (code no. M042) was used for  
6 double fluorescent immunostaining at week 1 and continued for up to 5 weeks. Figure  
7 4(a) shows that osteocalcin is expressed near FBXW2, but it is localized to small regions.  
8 Figure 4(b) shows blood vessels at 1 week and FBXW2 expression in blood vessels.  
9 Figure 4(c)–(f) show high magnification of bovine periosteal cells, which may synthesize  
10 osteocalcin. Shape of osteocalcin-resembled cells (Fig 4(c),(d)), stick (Fig 4(e)), and  
11 FBXW2 (Fig 4(f)). Figure 5a-e show the expression of osteocalcin along multiple edges  
12 of the periosteum; expression of osteocalcin increased with respect to observations at 1  
13 week. At 2 weeks, FBXW2 expression decreased along the edges of periosteum, while  
14 cells and osteocalcin remained the same (Fig 5(a)–(e)). Expression of RANKL, which  
15 was used as a negative control, was absent (Fig 5(f)). Osteocalcin expression along the  
16 edges increased at 3 weeks (Fig 6(a)) and multiple layers of periosteum-derived cells  
17 appeared (Fig 6(b)). Osteocalcin appeared in the regions where FBXW2 levels had  
18 decreased (Fig 6(c)). At 4 weeks, a stick of osteocalcin poked out of the periosteum with

1 multiple layers of periosteum-derived cells (Fig 7). At 5 weeks, FBXW2 disappeared  
2 from the regions where periosteum-derived cells had appeared (Fig 8(a),(b)), while cells  
3 and osteocalcin remained unaffected (Fig 8(b)–(d)).

4 **Fig 4.** (a)-(f) Double fluorescent immunostaining of periosteum at 1 week. Scale bar:  
5 100µm. Osteocalcin: green, FBXW2: red, DAPI: blue

6 **Fig 5.** (a)-(e) Double fluorescent immunostaining of periosteum at 2 weeks. Scale bar:  
7 100 µm. Osteocalcin: green, FBXW2: red, DAPI: blue; (f) negative control, RANKL:  
8 green

9 **Fig. 6.** (a)-(c) Double fluorescent immunostaining of periosteum at 3 weeks. Scale bar:  
10 100 µm. Osteocalcin: green, FBXW2: red, DAPI: blue

11 **Fig 7. Double fluorescent immunostaining of periosteum at 4 weeks.** Scale bar: 100µm.  
12 Osteocalcin: green, FBXW2: red, DAPI: blue

13 **Fig 8.** (a)-(d) Double fluorescent immunostaining of periosteum at 5 weeks. Scale bar:  
14 100 µm. Osteocalcin: green, FBXW2: red, DAPI: blue

## 15 **Discussion**

16 This study highlights the role of FBXW2. The study revealed that FBXW2 is  
17 located in the bone, cambium layer, periosteum, and capillary. Conversely, FBXW2  
18 disappeared around the bulk of osteocalcin and periosteum-derived cells. On day 0,



1 FBXW2 was expressed in the capillary, but osteocalcin was not expressed around the  
2 capillary (Fig 3). At day 5, the capillary-like structure collapsed with FBXW2 (Fig 9).  
3 Leonard et al. [14] reported that Sirtuin 1 levels decreased in the internal artery of diabetic  
4 patients, while osteocalcin levels increased. Therefore, the relationship between FBXW2  
5 and vascular calcification needs to be studied. My observation of FBXW2 expression  
6 from day 0 to 5 weeks indicates that FBXW2 plays a role in maintaining tissue shape. It  
7 can be hypothesized from Fig 8(a) that FBXW2 maintains the shape of the periosteum  
8 and prevents cells from migrating out, and the disappearance of FBXW2 causes migration  
9 of periosteum-derived cells out of the periosteal tissue. I further hypothesized that at day  
10 5, the capillary that was no longer needed was disassembled along with FBXW2. In 2015,  
11 Hirashima et al. [15] revealed the anchoring structure of the calvarial periosteum.  
12 However, the components of the anchoring structure are not clear. In this study, the  
13 cambium layer of the periosteum needs FBXW2 to adhere to the bone. Bone is a hard  
14 tissue and requires less FBXW2 than the cambium layer. Sun's group investigated the  
15 relationship between FBXW2 and lung cancer cells [16,17]. They reported that FBXW2  
16 suppressed lung cancer cell migration and invasion by inhibiting the escape of these cells.  
17 **Fig 9. Double fluorescent immunostaining of periosteum at day 5.** Scale bar: 100µm.  
18 Osteocalcin: green, FBXW2: red, DAPI: blue

1           The relationship between FBXW2 and osteocalcin has not been established. In  
2   2018, Akiyama [13] reported that FBXW2 was localized with osteocalcin first. Recent  
3   studies have reported a relationship between osteocalcin and diabetes [14,18-20]. In this  
4   study, osteocalcin was localized in the bone, but RANKL was not. Osteocalcin is an  
5   important bone matrix protein [21] and a typical biomarker for bone maturation [22],  
6   whereas RANKL is crucial for osteoclastogenesis [23–25]. Thus, in this study, RANKL  
7   was not observed as this period did not involve osteoclast formation. Two antibodies for  
8   osteocalcin, a mouse monoclonal antibody for osteocalcin (No. sc-376835), and  
9   monoclonal antibody to bovine osteocalcin (code no. M042), were used. To compare with  
10  the RANKL antibody, no. sc-376835 was used, but for double fluorescent  
11  immunostaining, code no. M042 was used to obtain a sharp contrast. No. sc-376835 is of  
12  mouse origin, whereas code no. M042 is bovine osteocalcin, the latter being more  
13  sensitive. On day zero, osteocalcin was not expressed in the periosteum. At one week, a  
14  small amount of osteocalcin appeared with FBXW2 (Fig 4(a)). In a previous study, at five  
15  weeks, both FBXW2 and osteocalcin formed long and thin tubes [13]. It is hypothesized  
16  that osteocalcin-forming cells have an affinity for FBXW2. If FBXW2 has a role in tissue  
17  shape maintenance, it can be assumed that osteocalcin was synthesized on a template of  
18  FBXW2. At 2 weeks, the bulk of osteocalcin was separated from FBXW2 with the

1 exception of tube formation (Fig 5(a)–(c)). In Fig 5(f), the antibody for RANKL was used  
2 as a negative control under the same conditions (mouse IgG<sub>1</sub> concentration and treatment  
3 time were 4 µg/mL and 4 h, respectively). At three weeks, periosteum-derived cells  
4 started to migrate out of the periosteum (Fig 6b) and FBXW2 expression decreased at the  
5 root of periosteum-derived cells. At 4 weeks, osteocalcin burst out of the periosteum and  
6 fell onto periosteum-derived cells. At 5 weeks, multilayered periosteum-derived cells  
7 appeared and FBXW2 disappeared in periosteum-derived cells.

8           Akiyama et al. [7] reported that transplanted bovine periosteum-derived cells can  
9 form new bone. Osteoclasts were followed using TRAP [10] from periosteum-derived  
10 cells up to the formation of new bone. Therefore, RANKL, a biomarker of osteoclasts,  
11 may be expressed even after transplantation. An important characteristic of periosteum-  
12 derived cells is that they can form multilayered cell sheets *in vitro* without artificial  
13 scaffold materials [8]. In this study, primary cultured cells were investigated. However,  
14 secondary passage cells cannot form multilayered cell sheets (data not shown). In Fig 8(a),  
15 periosteum-derived cells seem to form multilayered cell sheets at the inner periosteum  
16 and then migrate out of the periosteum. Therefore, secondary passage cells without  
17 periosteum cannot form multilayered cell sheets. The important question here is whether  
18 a multilayered cell sheet on tissue culture dishes is formed in the periosteum or outside

1 it. Multilayered cell sheets of periosteum-derived cells can carry out scaffold-free bone  
2 regeneration [7]. FBXW2 in the periosteum may contribute to multilayered cell sheet  
3 formation. Simon et al. [26] reported that surgical stimulation of the periosteum by a sharp  
4 incision causes cambium cell proliferation and new bone formation. They concluded that  
5 the reason for new bone formation was an increase in the thickness of the cambium layer.  
6 However, my results suggest that FBXW2 and osteocalcin *in vitro* are also involved in  
7 bone formation *in vivo*. However, I cannot conclude whether FBXW2 and osteocalcin are  
8 directly or indirectly related. Other plural proteins may be related to FBXW2 and  
9 osteocalcin. FBXW2 is not known to have osteoblastic function. However, osteocalcin is  
10 a biomarker of osteoblasts, and signaling pathways for osteogenic differentiation with  
11 osteocalcin have been reported [27–32]. In the future, signaling between FBXW2 and  
12 osteocalcin should be investigated. Determination of the osteoblastic role of FBXW2 may  
13 provide clues for the treatment of osteoporosis.

14

## 15 **Conclusion**

- 16 1. FBXW2 has a role in maintenance of tissue shape and synthesis of osteocalcin.
- 17 2. The disappearance of FBXW2 results in the release of periosteum-derived cells, while  
18 osteocalcin remains unaffected.

1

## 2 **Author contribution statement**

3 Mari Akiyama designed and performed the experiments, analyzed and  
4 interpreted the data, and wrote the paper.

5

## 6 **Competing interest statement**

7 The author declares no conflict of interest.

8

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14

Periosteum (Day 0 - 5weeks)

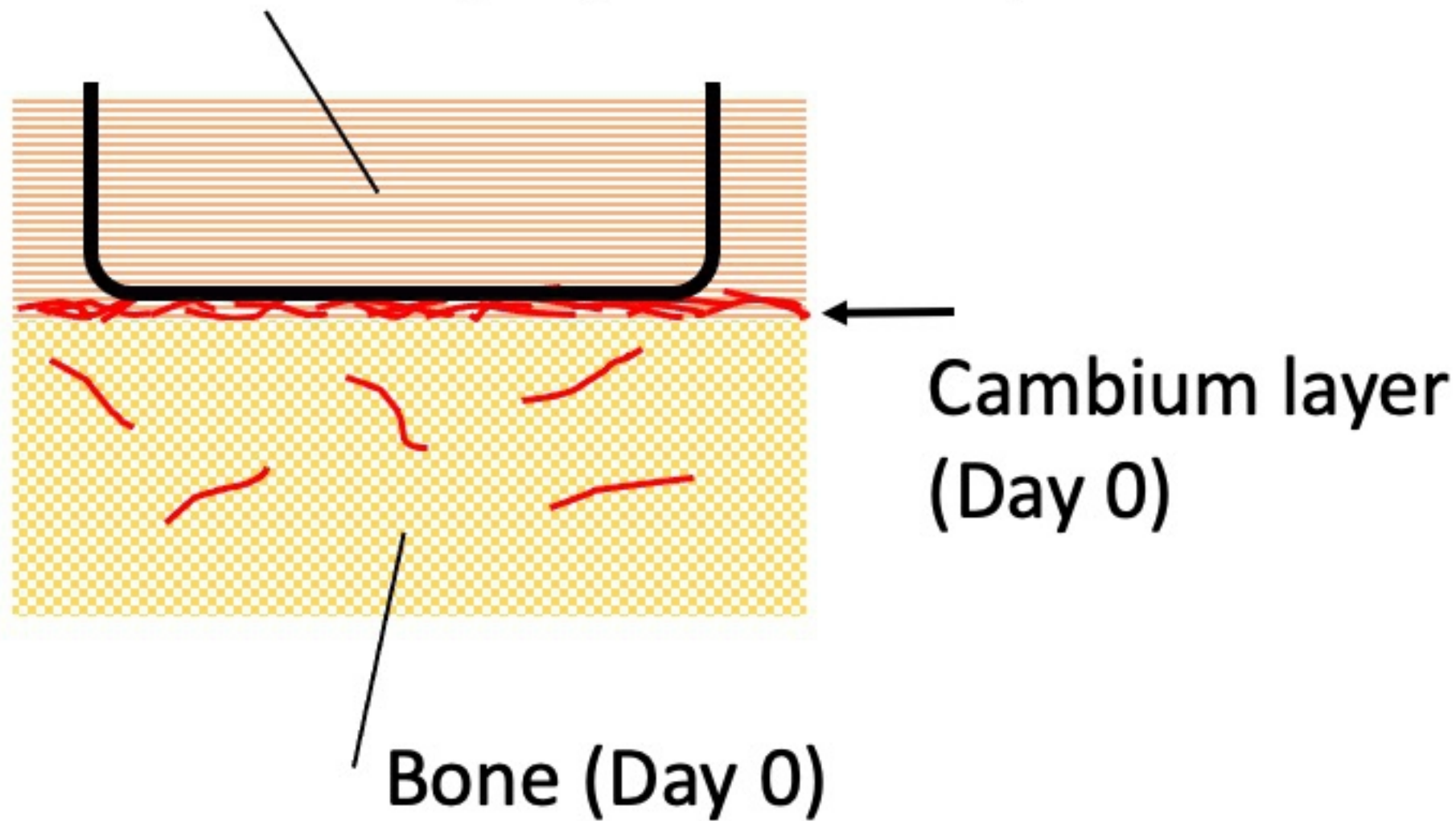
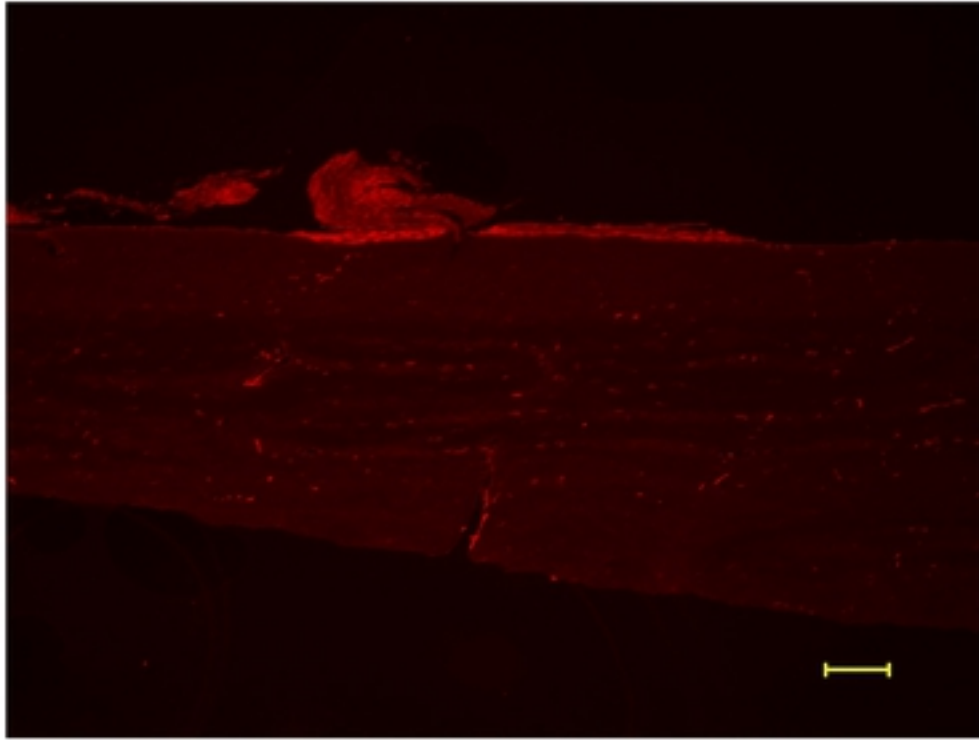
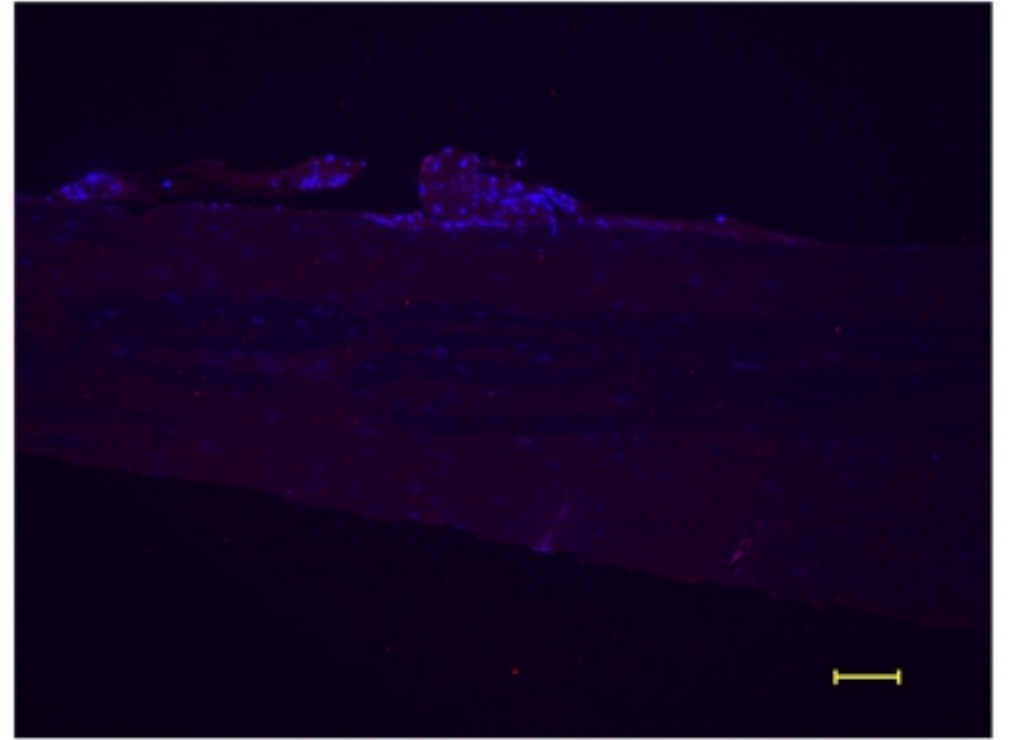


Figure 1

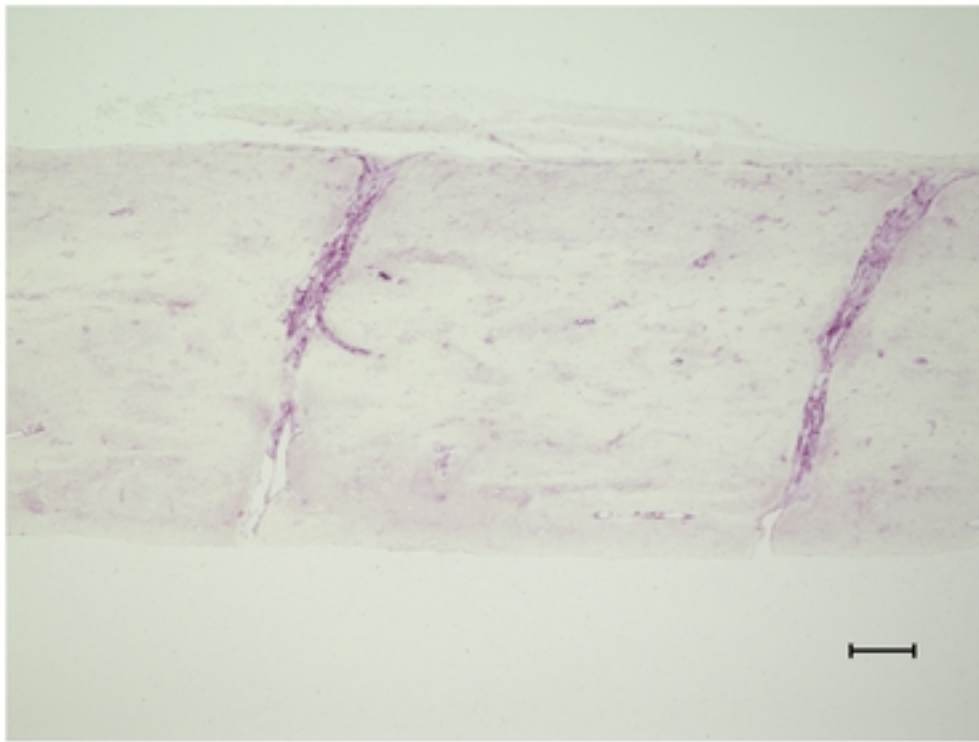
a



b



c



d

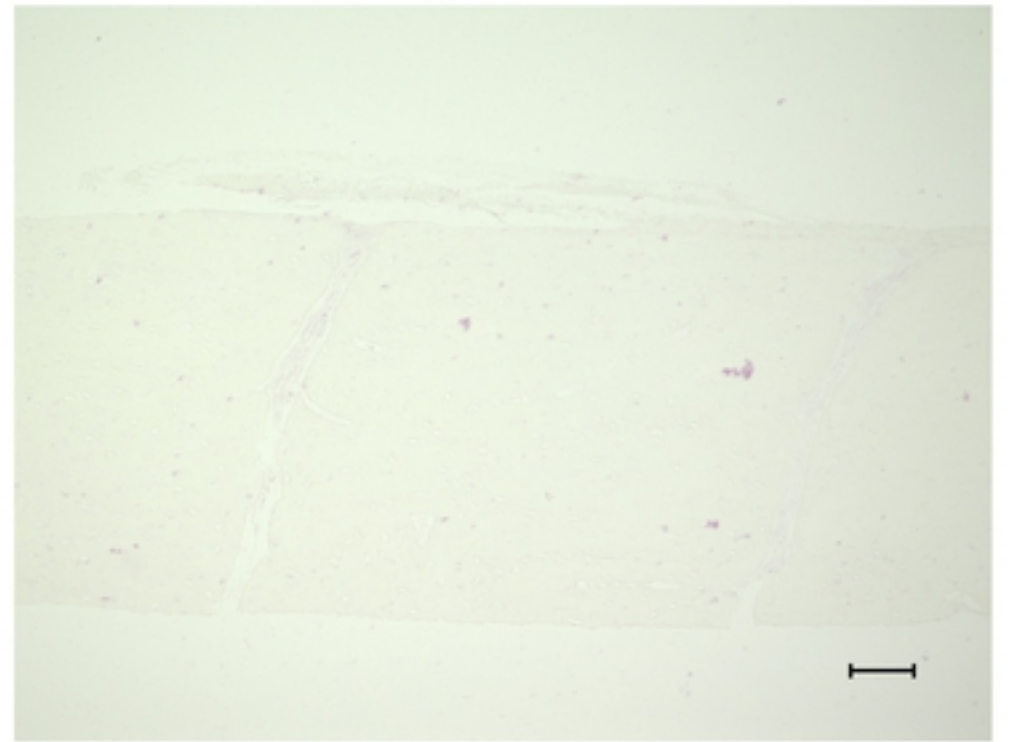
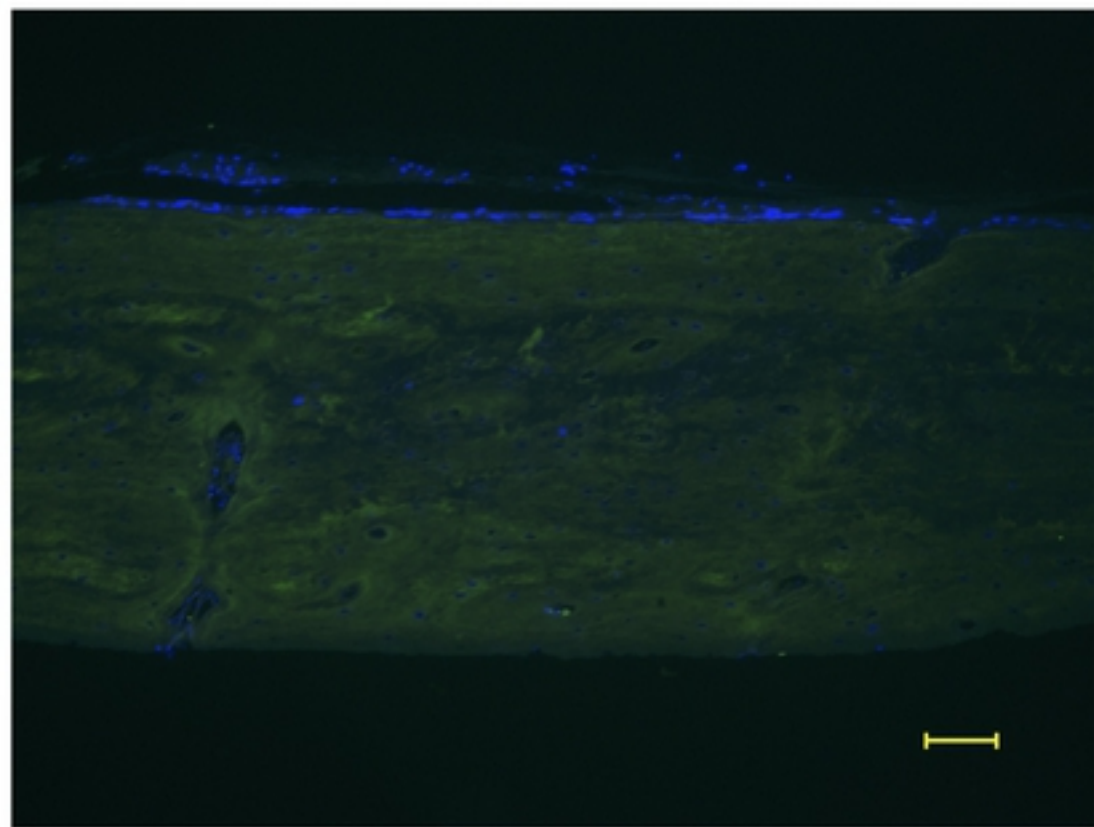


Figure2a-d

e



f

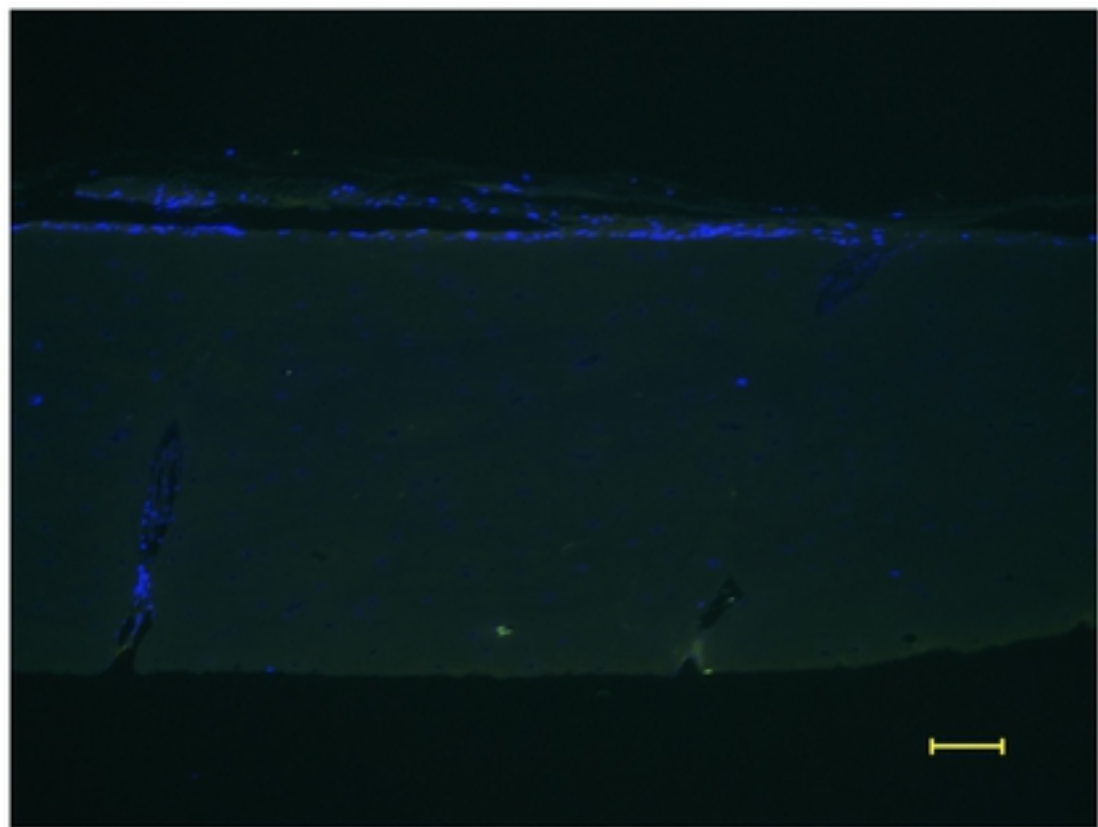


Figure 2e,f

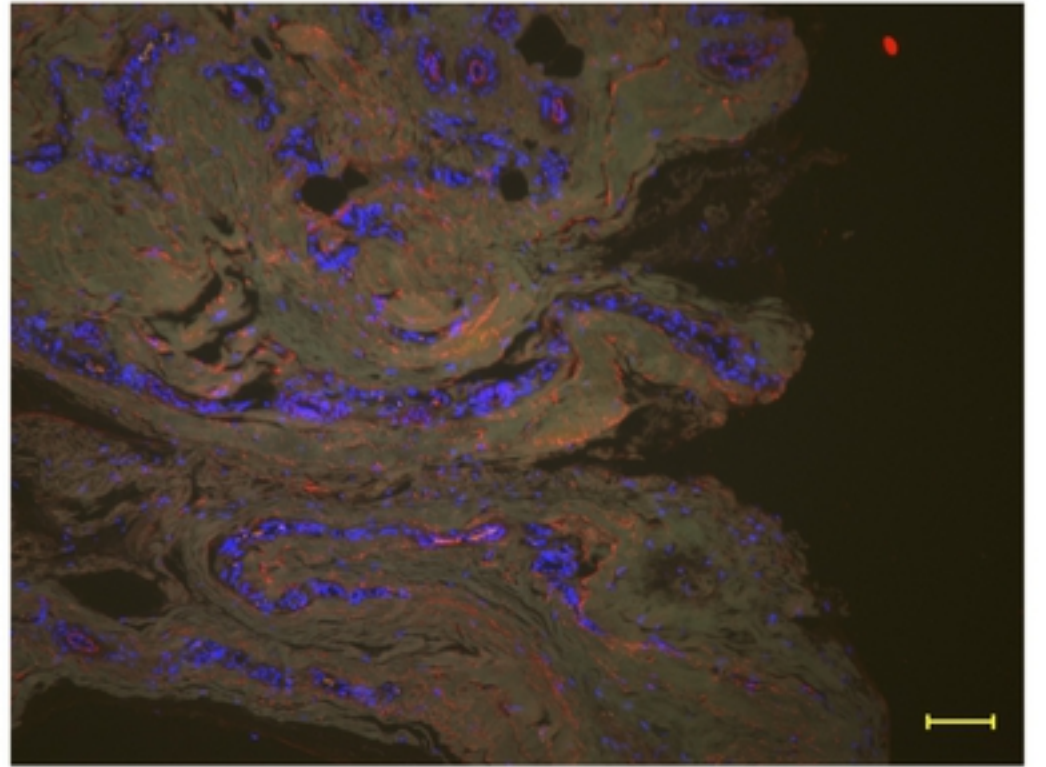
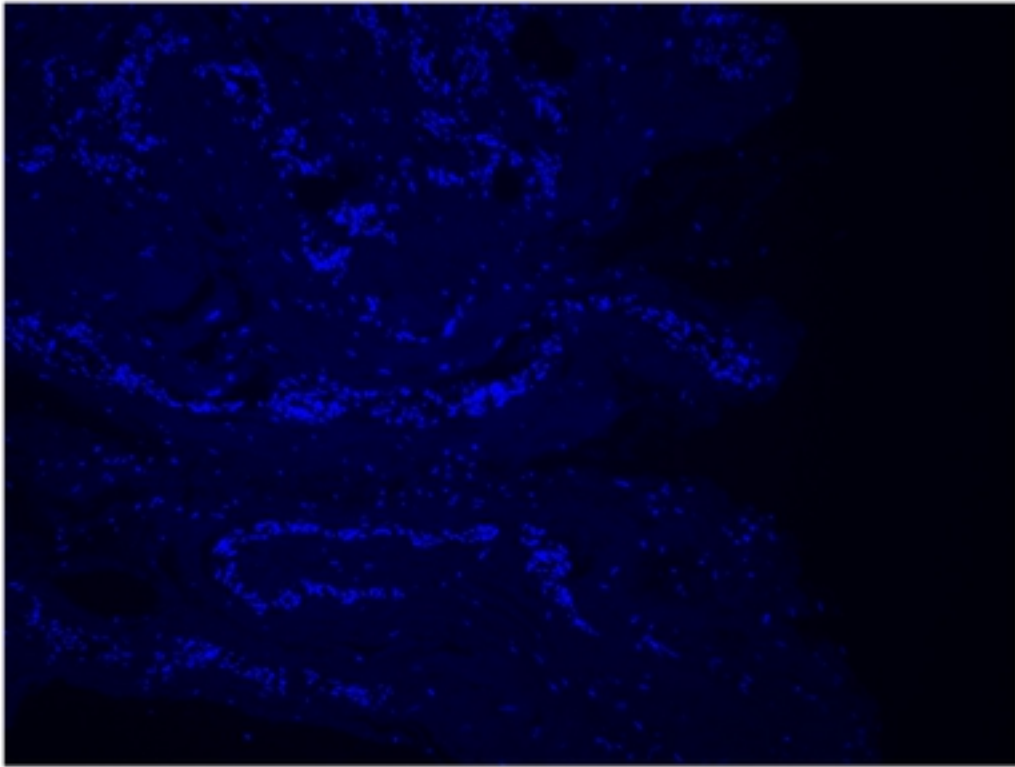
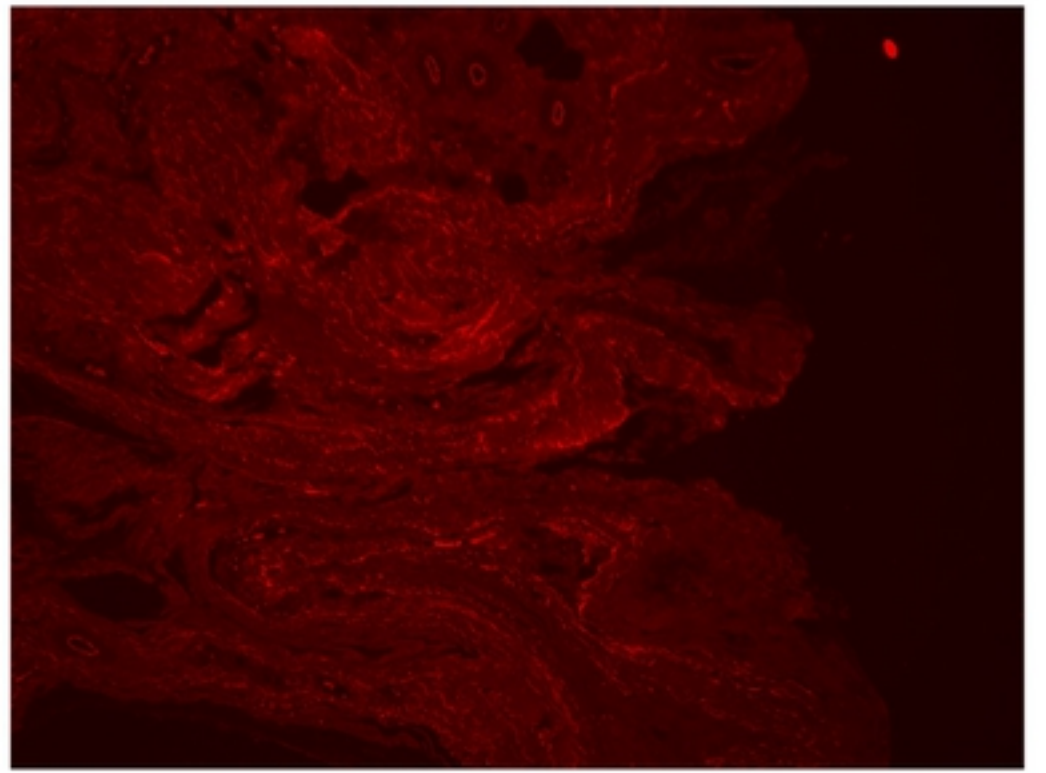
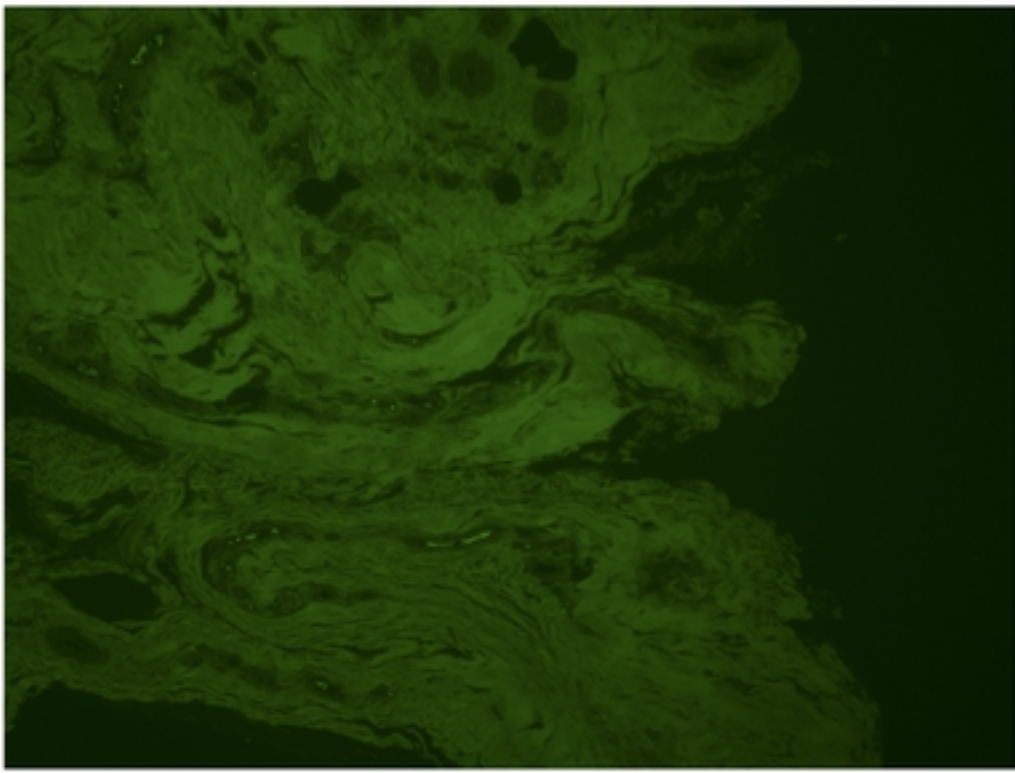


Figure3



a

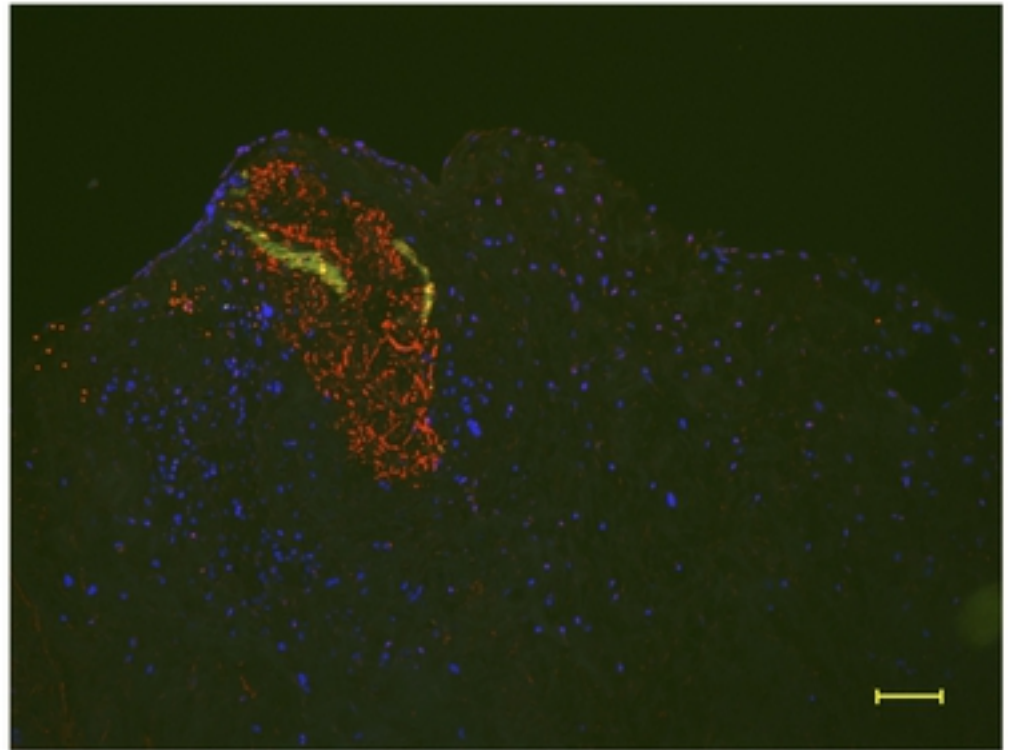
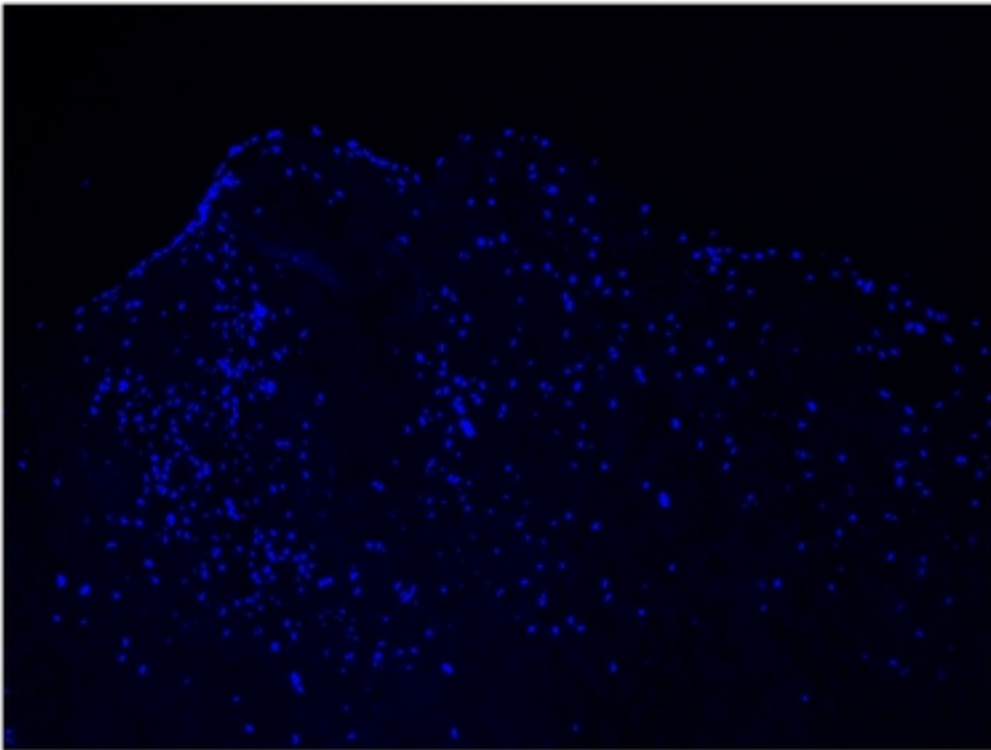
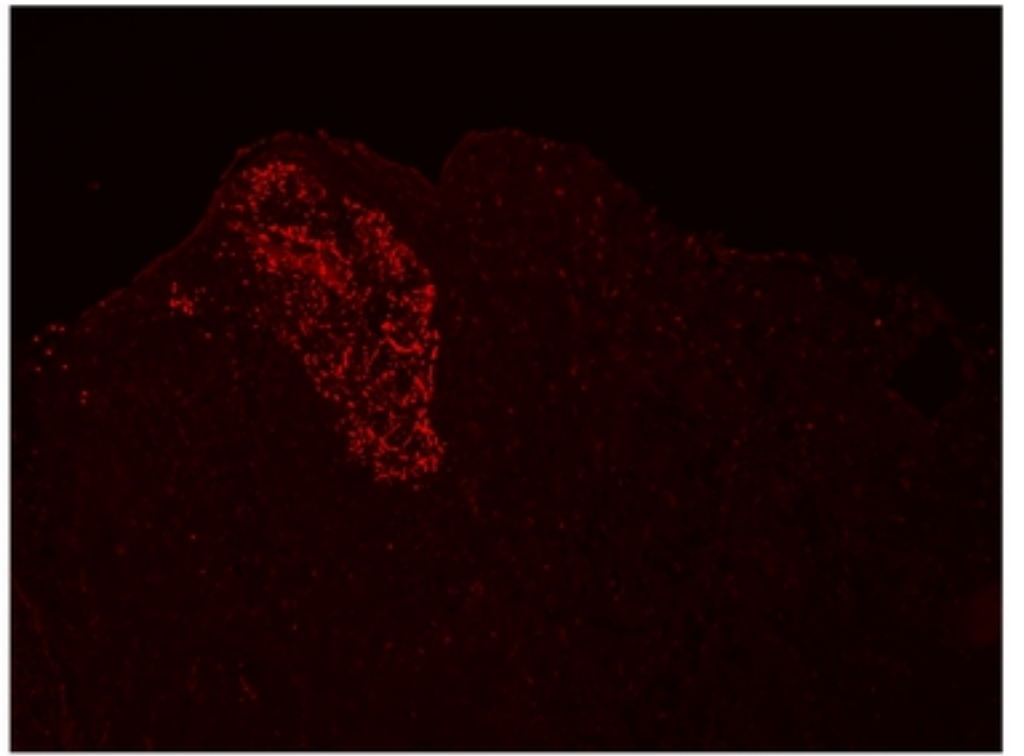
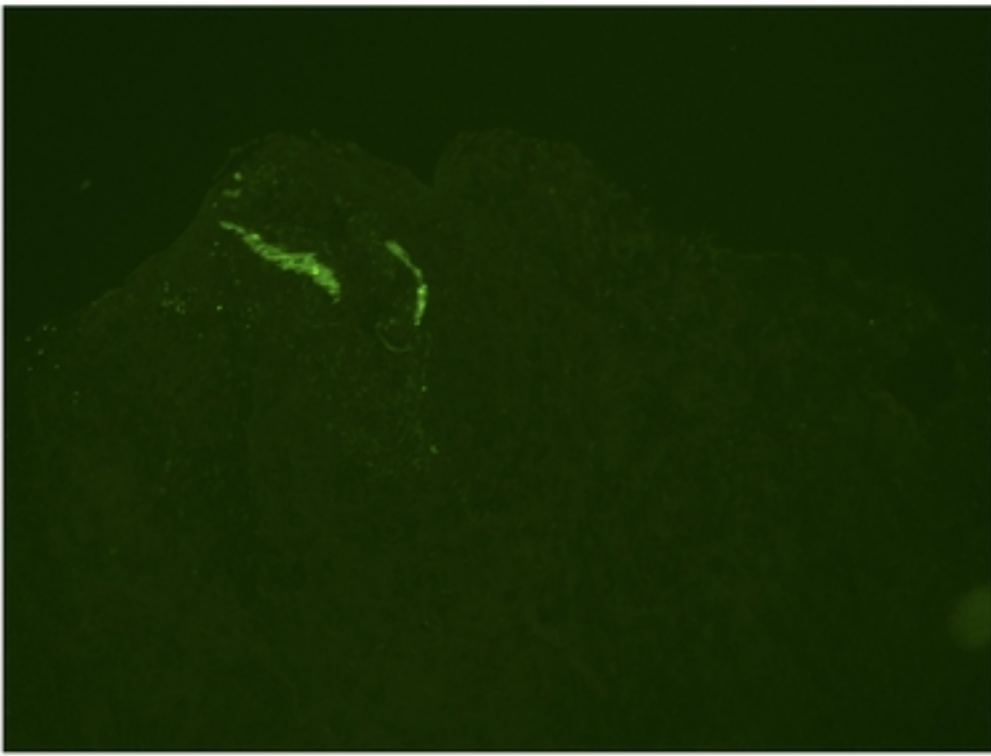


Figure4a

b

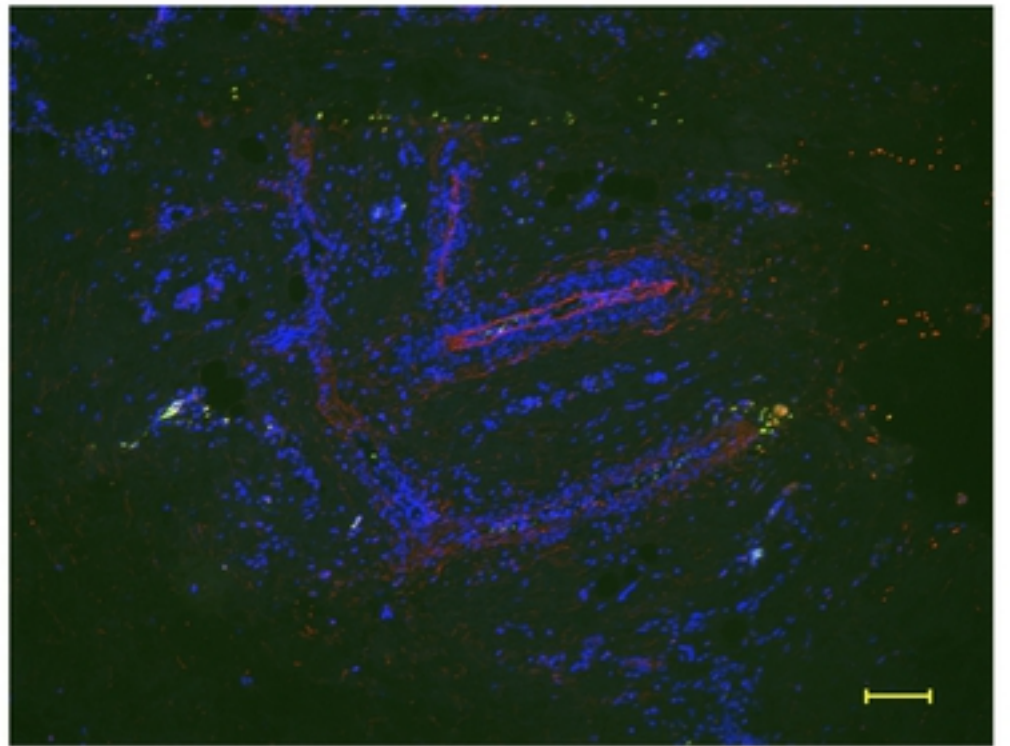
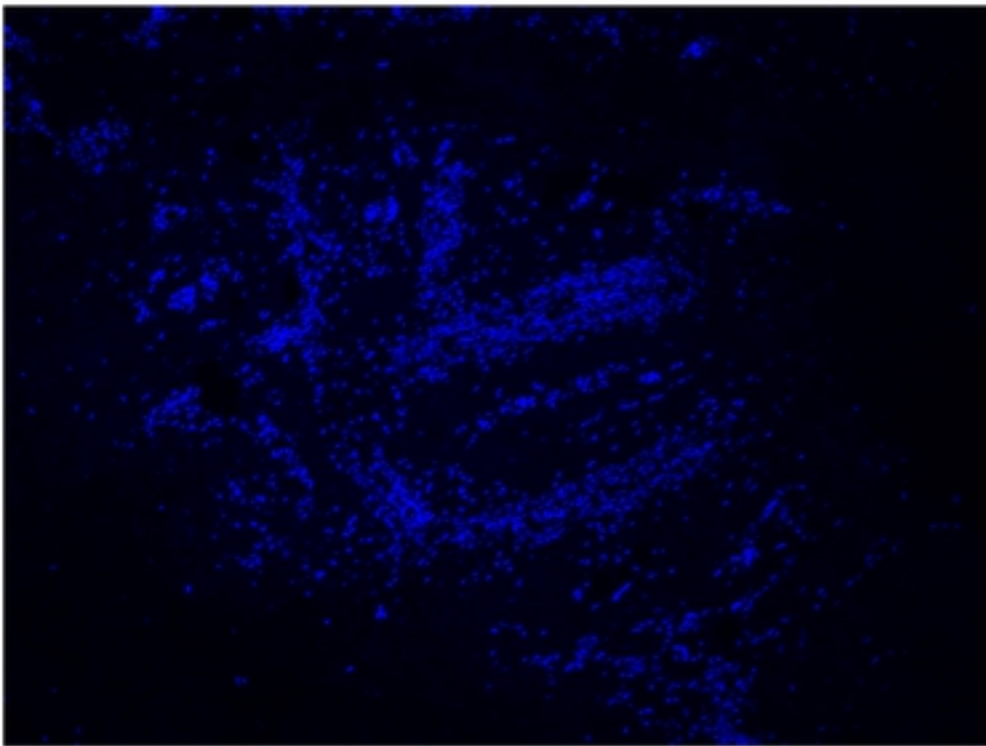
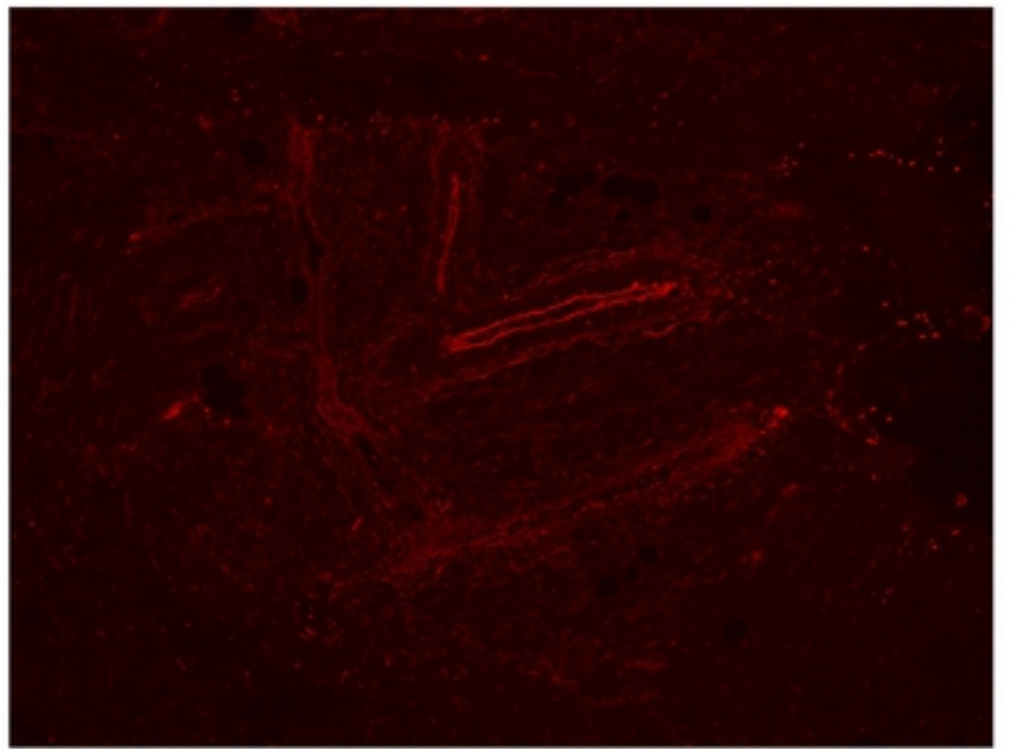
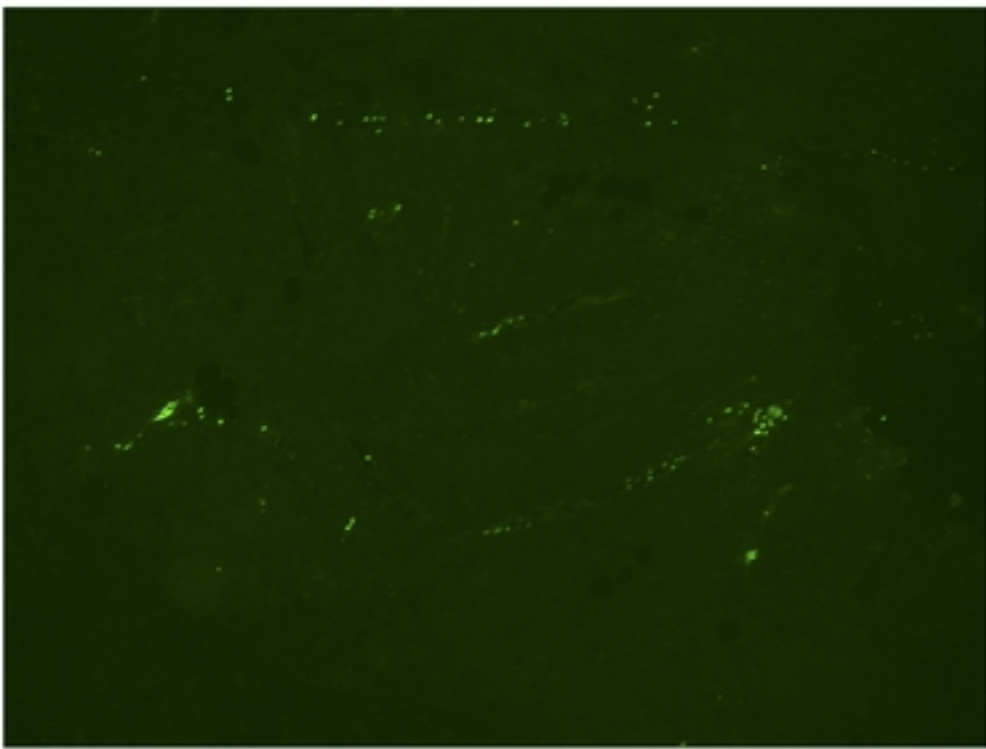


Figure4b

c

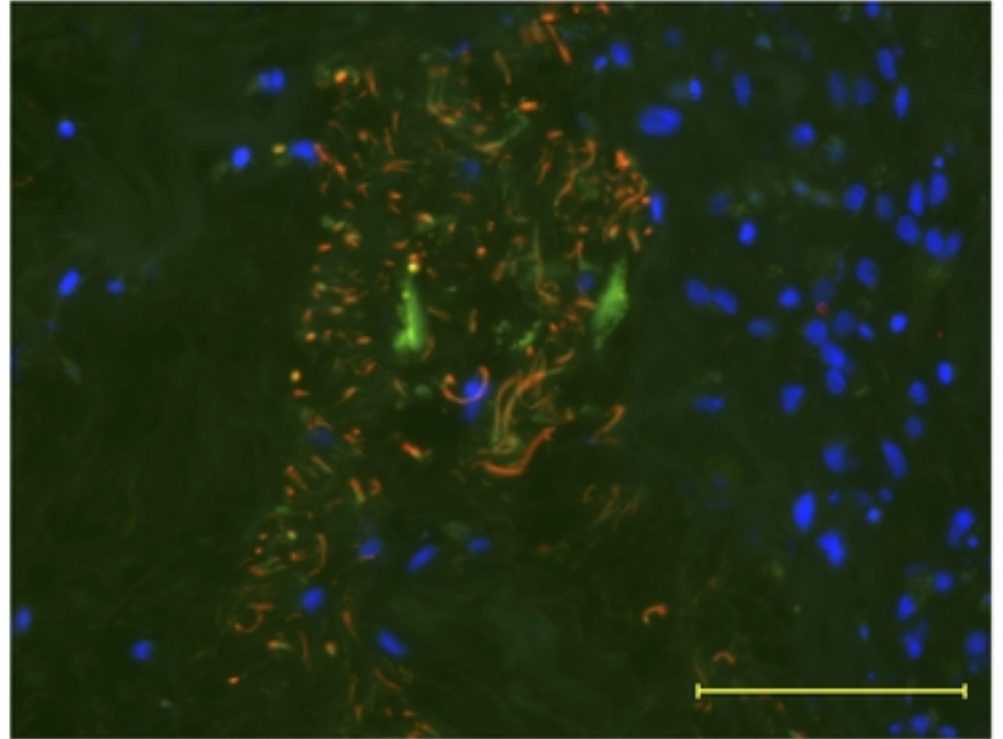
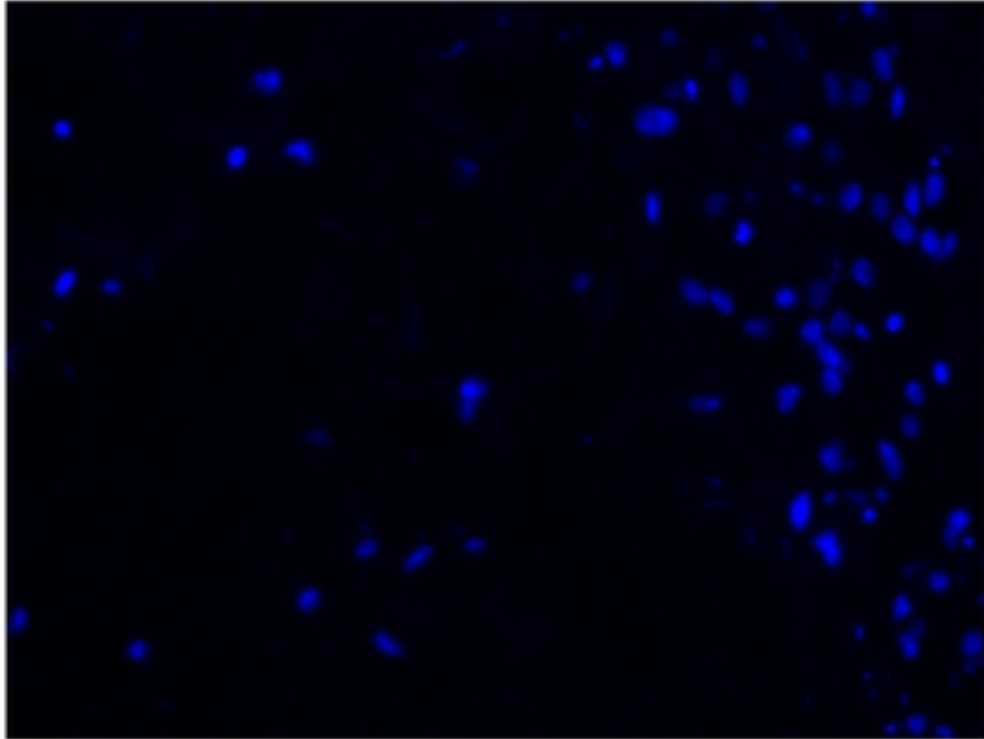
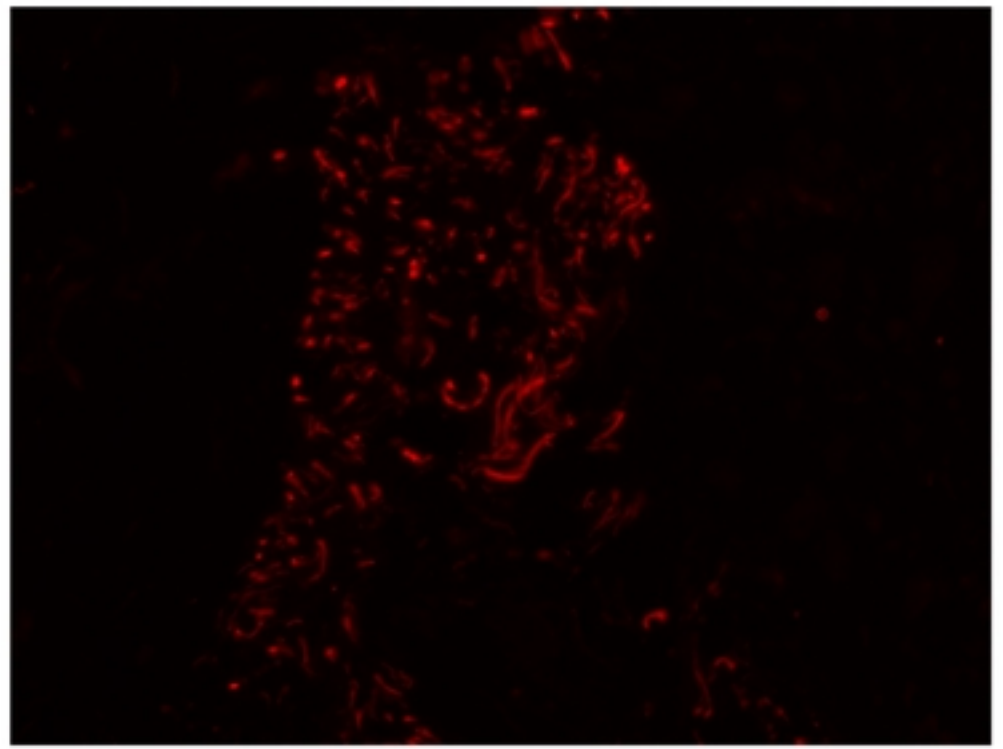
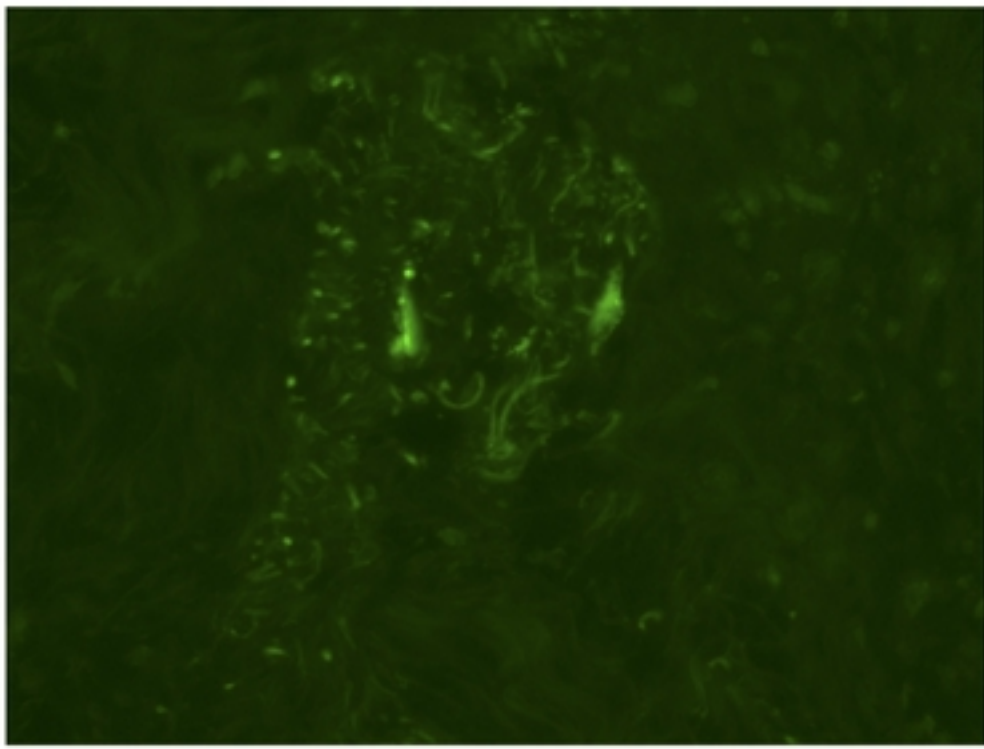


Figure4c

d

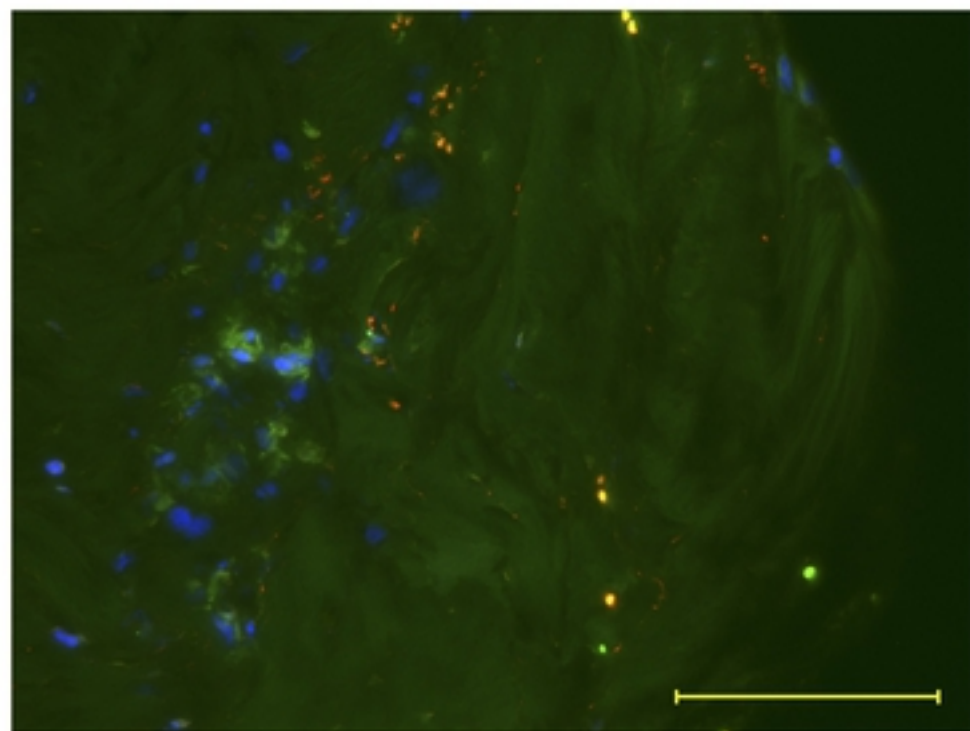
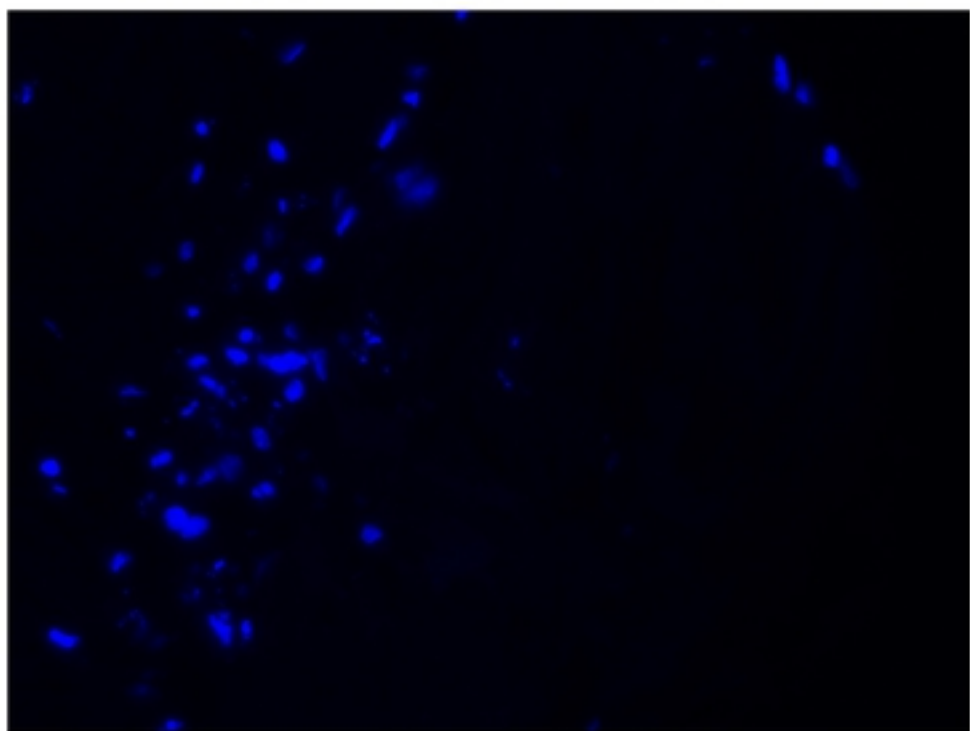
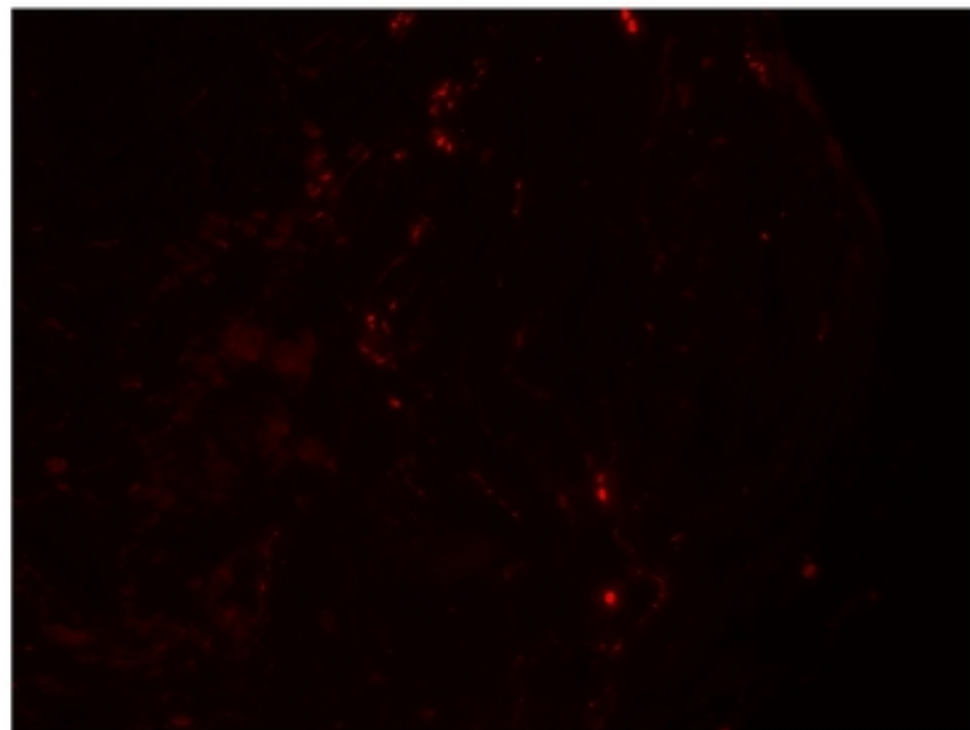
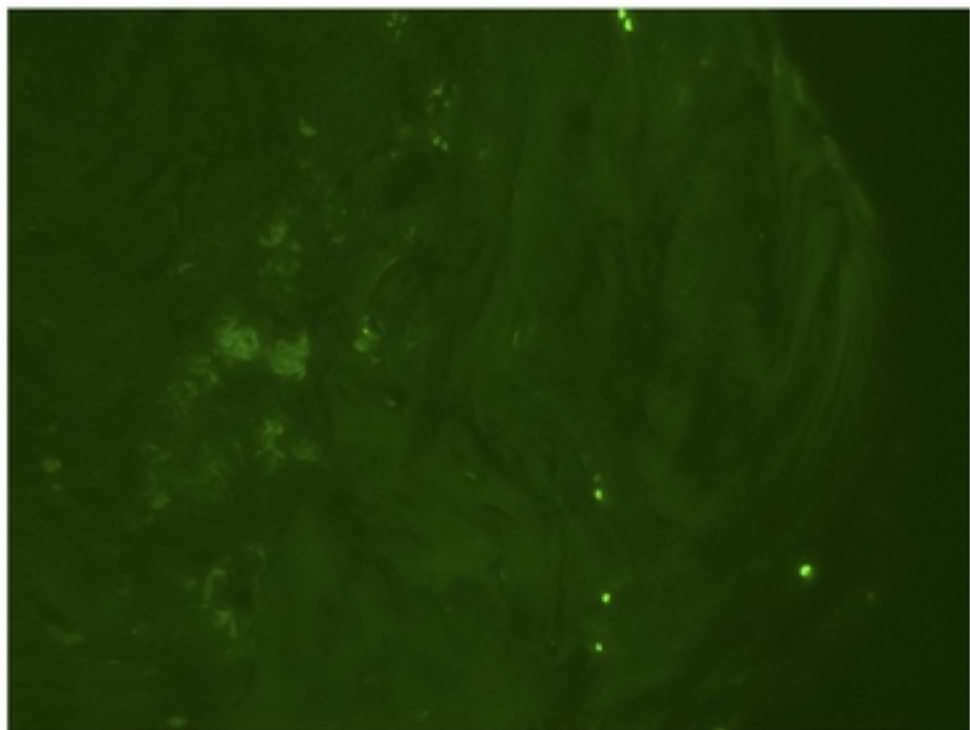


Figure4d

e

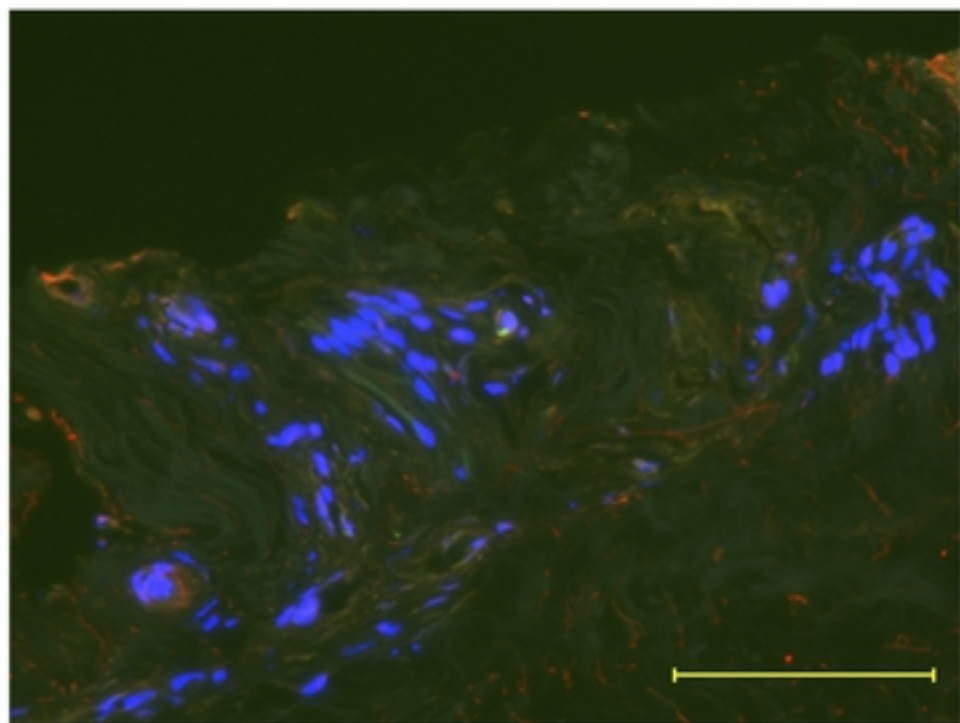
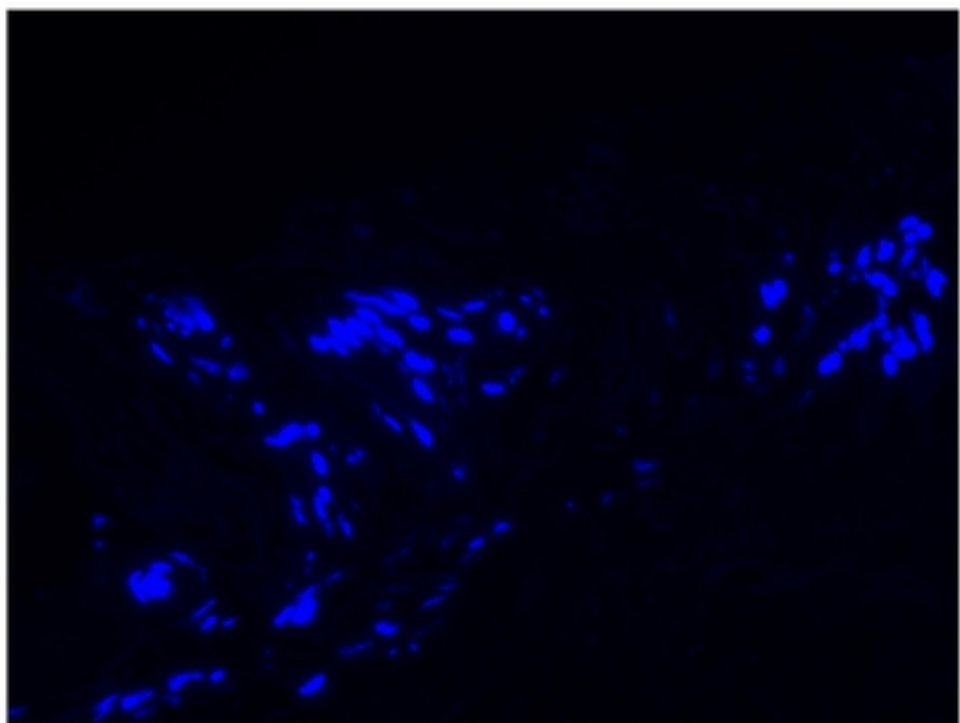
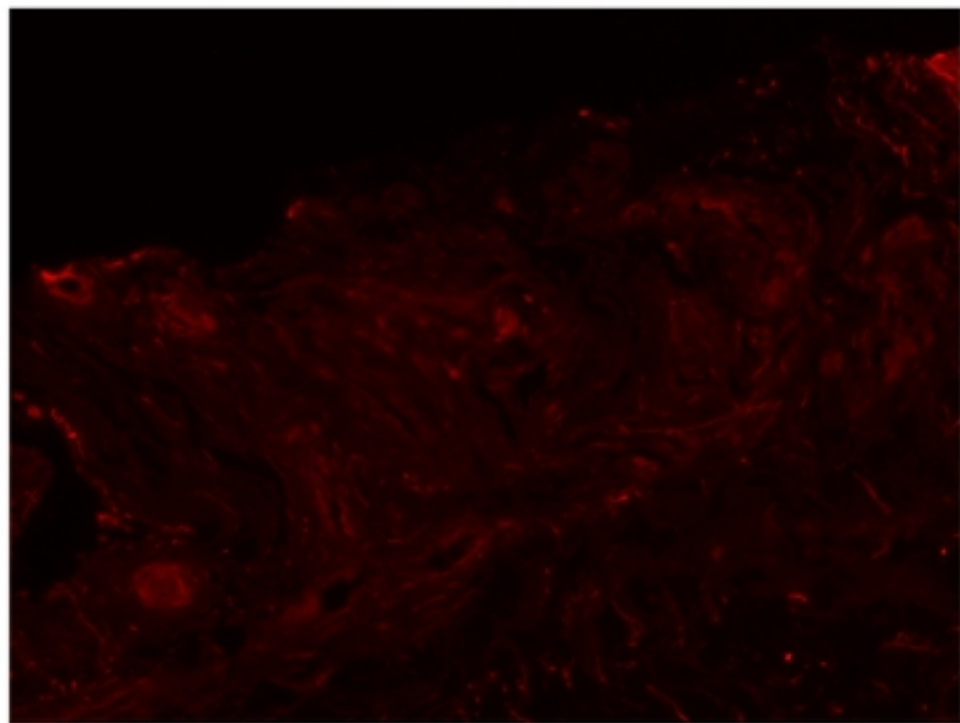
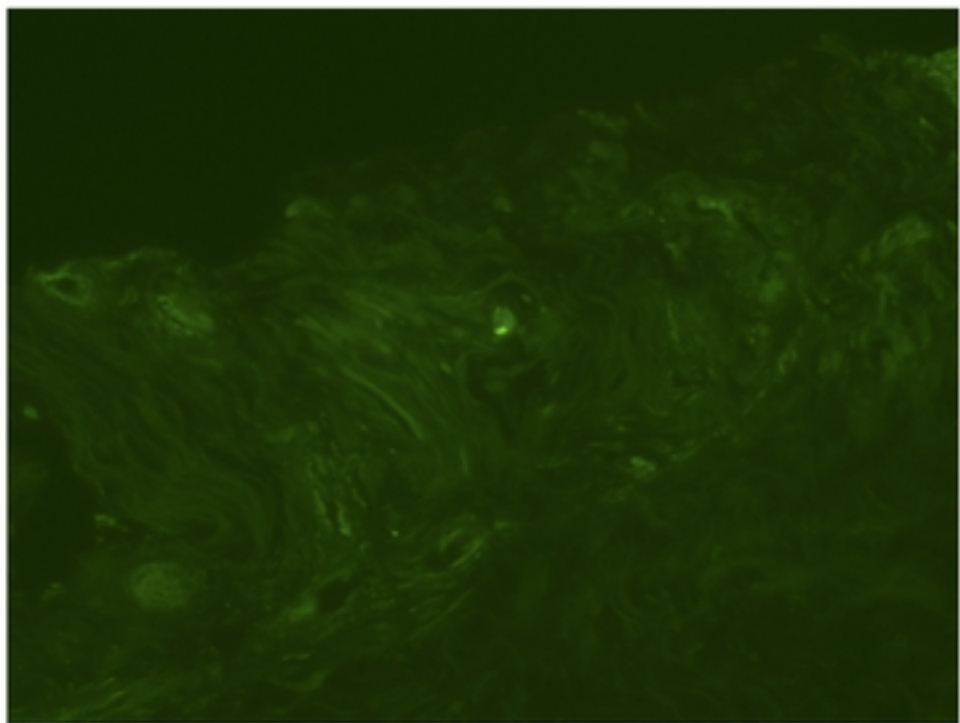


Figure4e

f

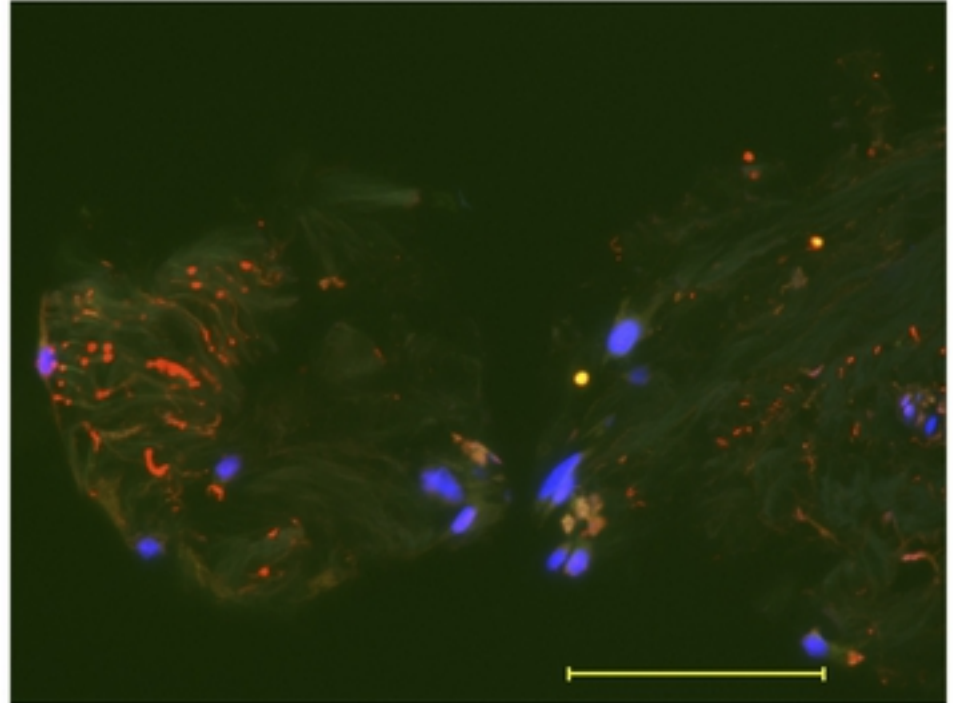
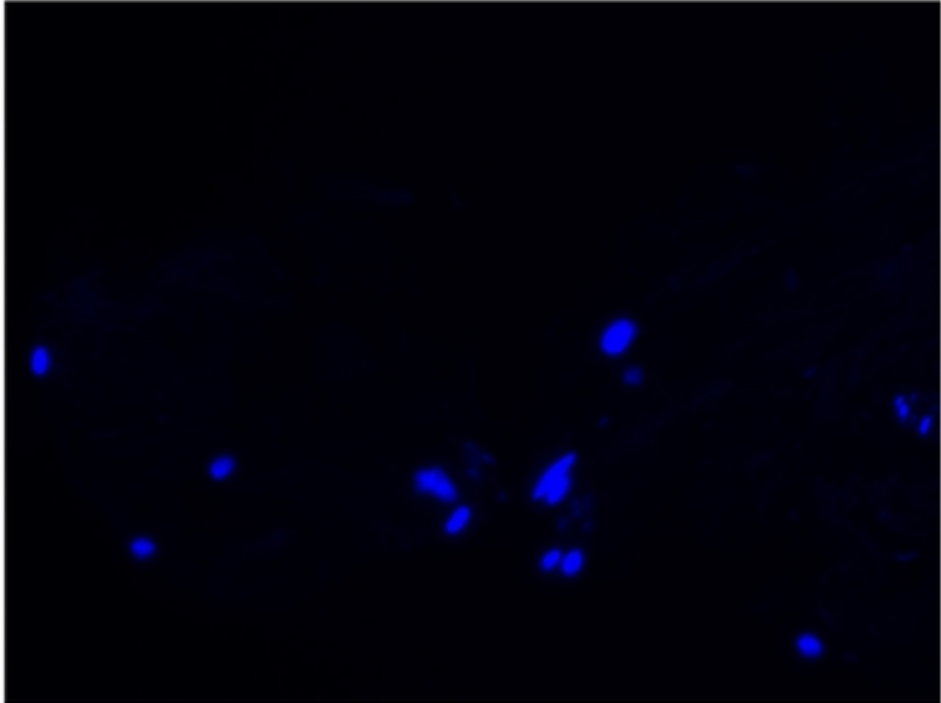
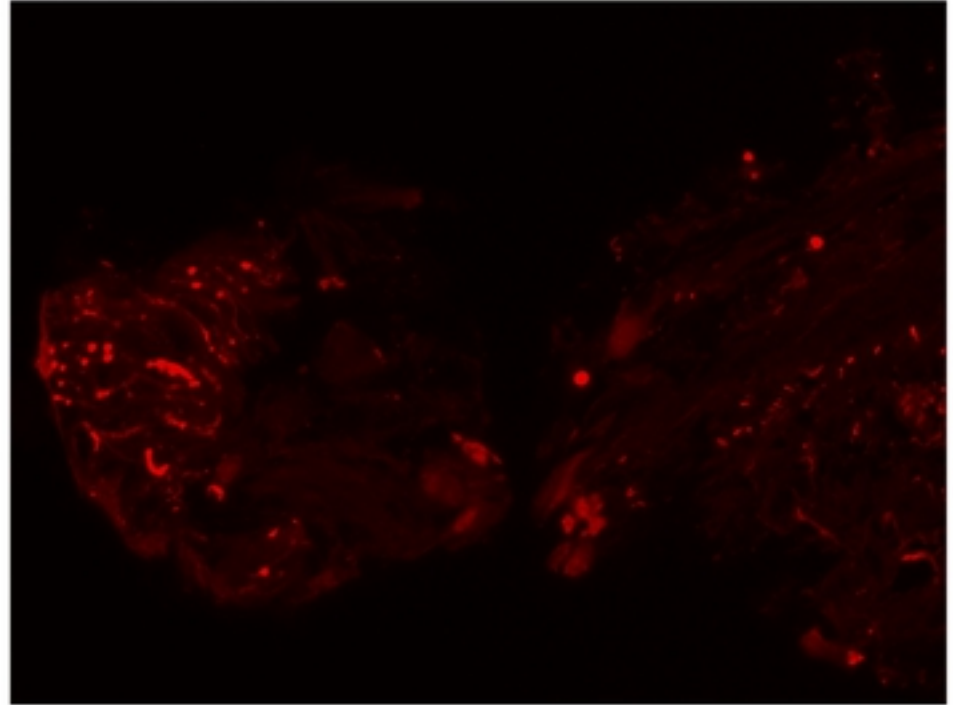
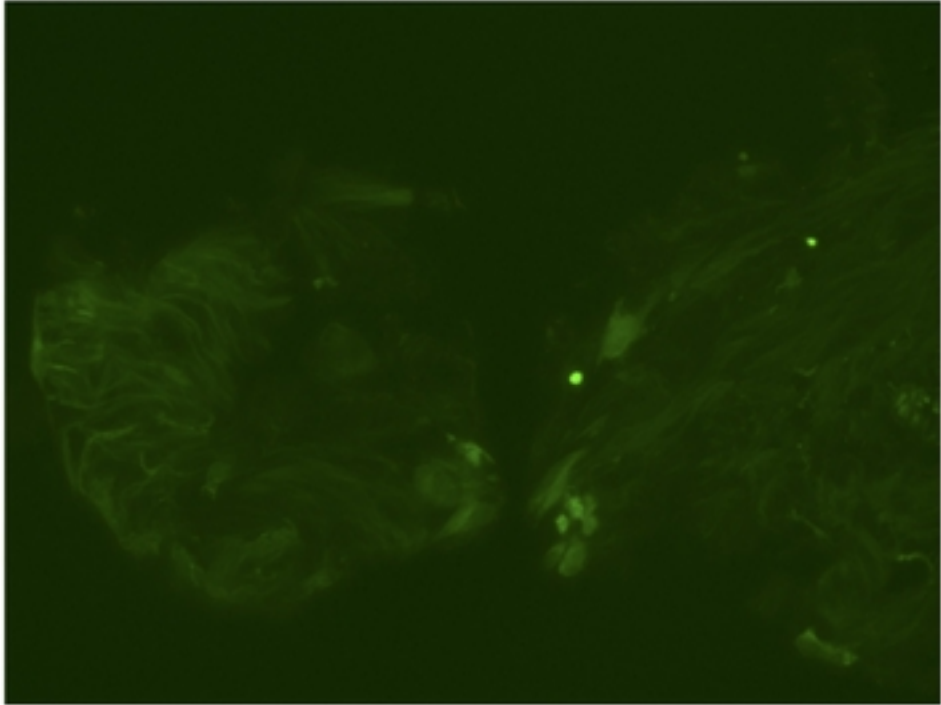


Figure4f

a

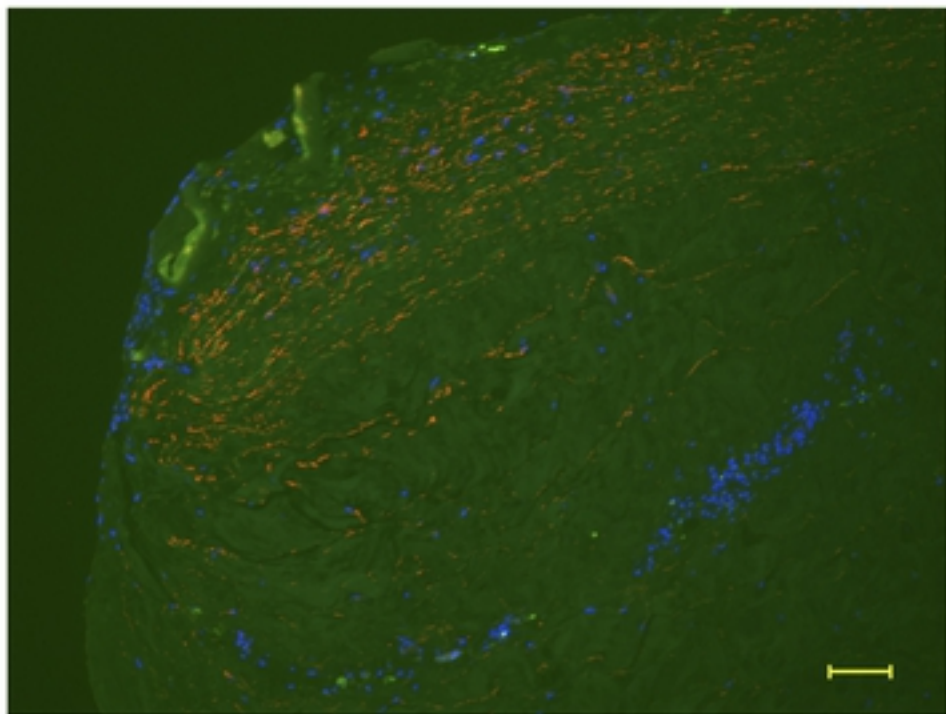
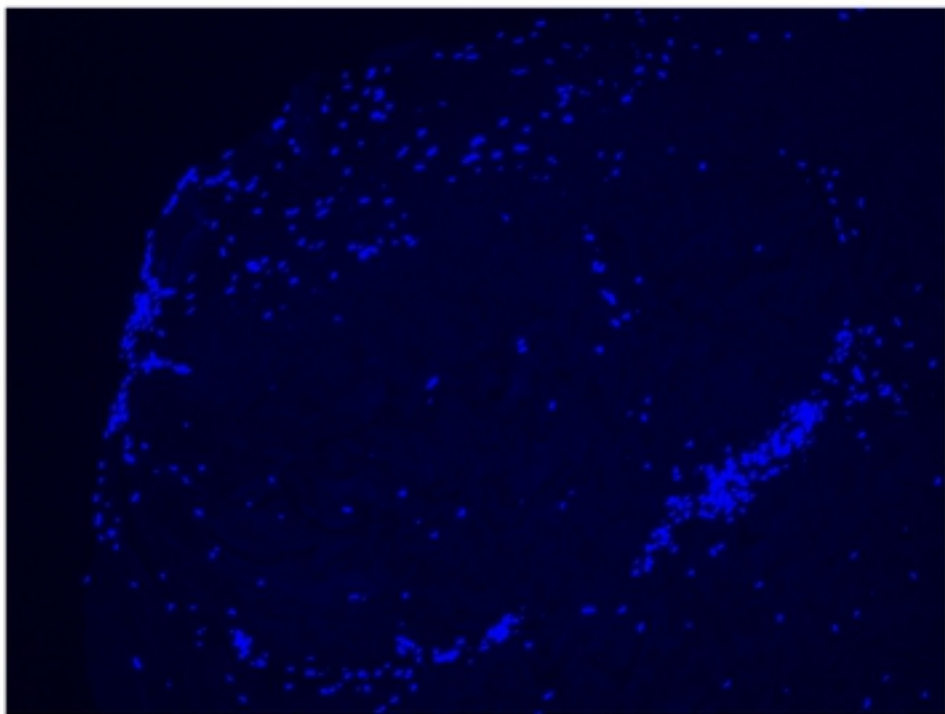
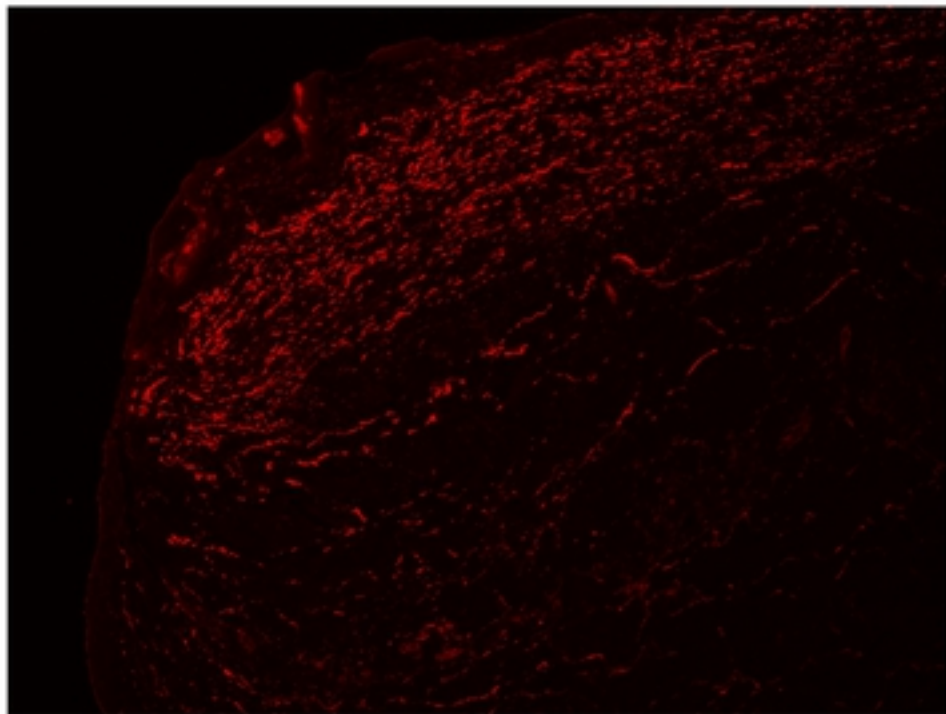
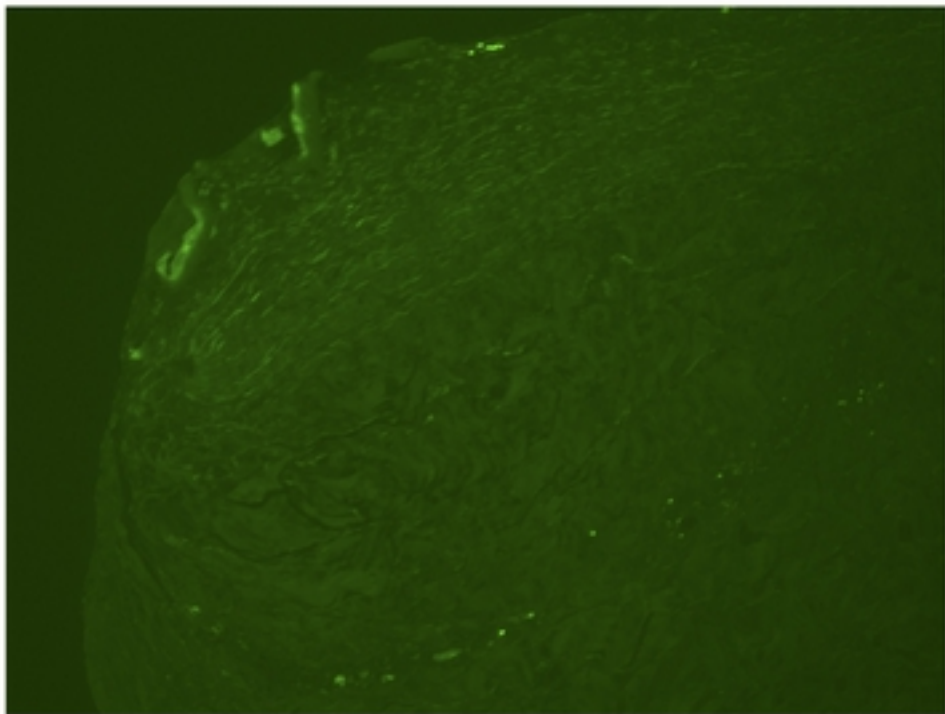


Figure5a

b

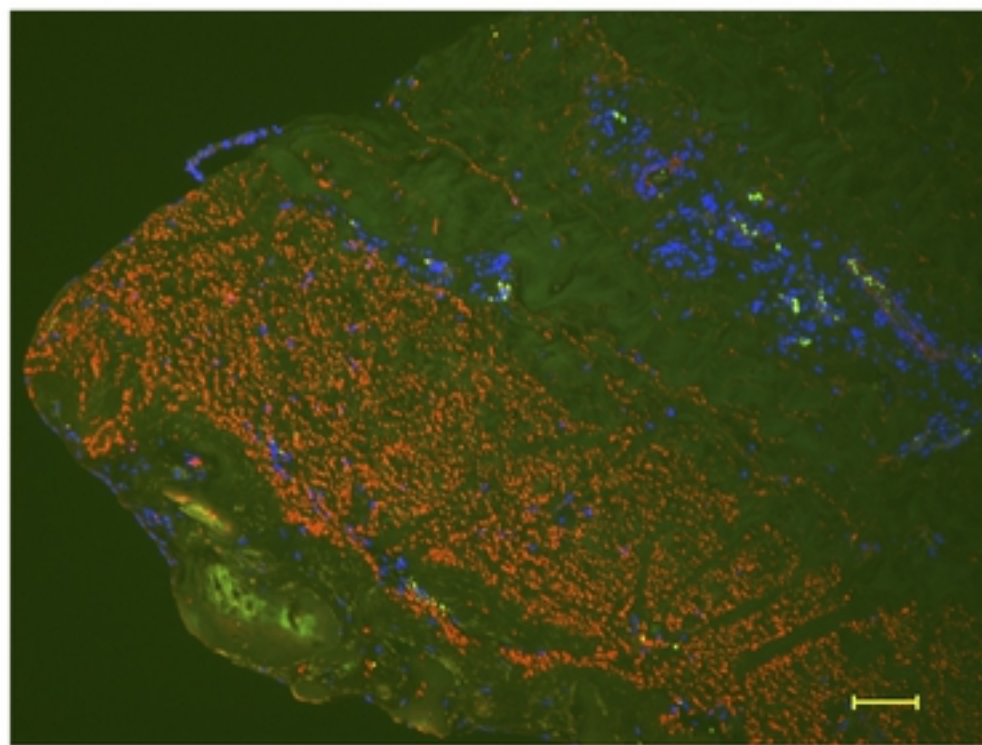
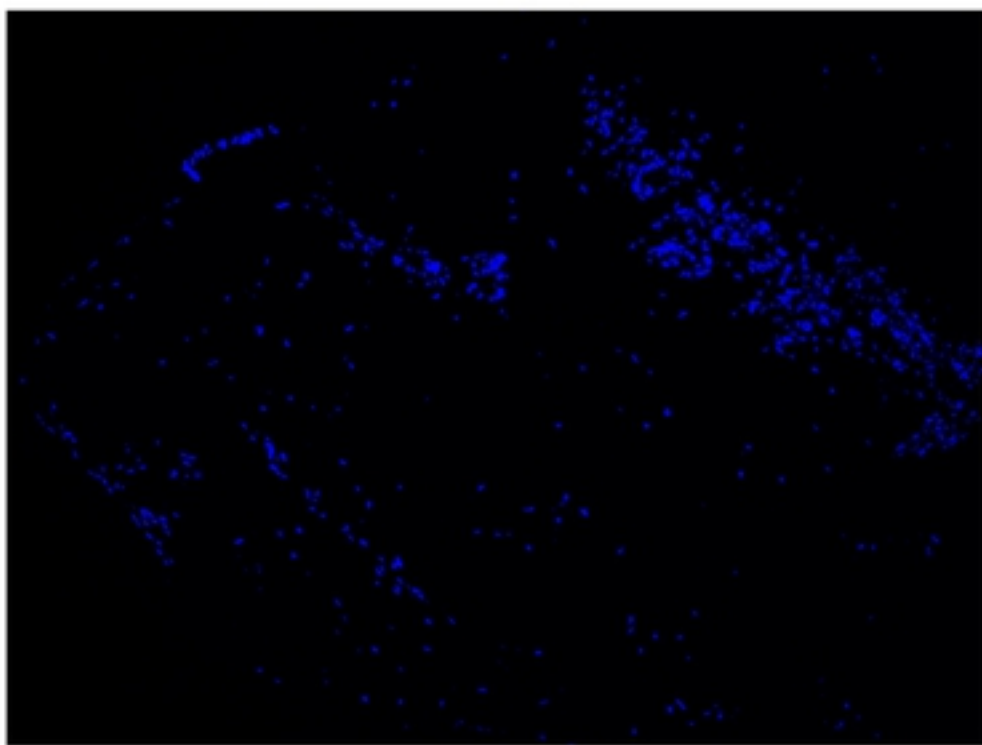
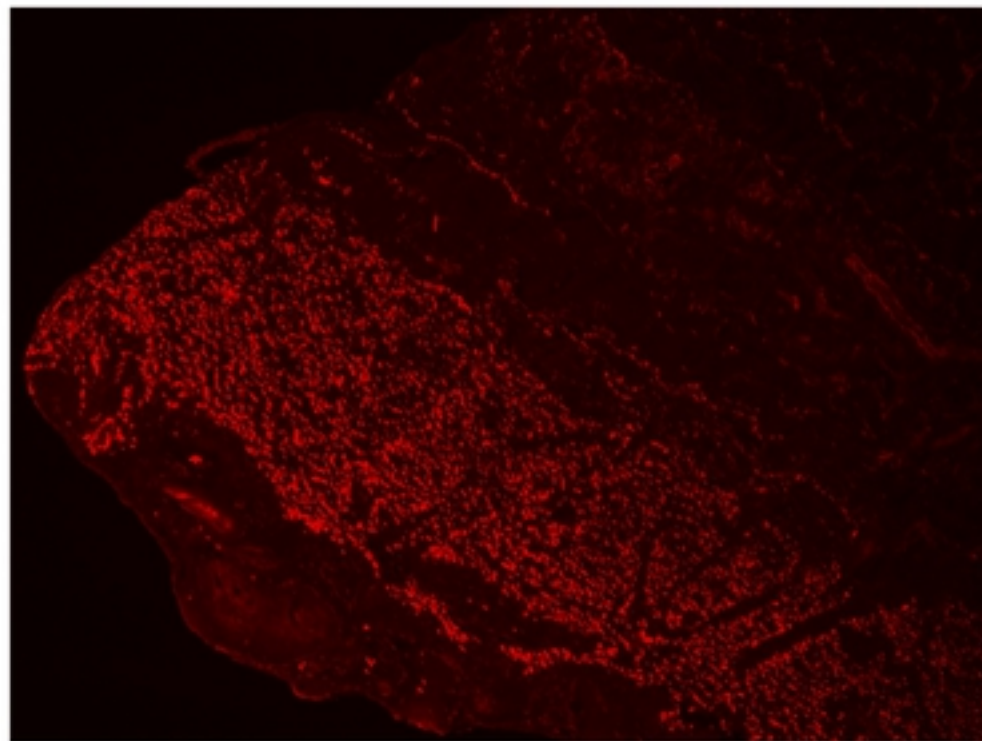
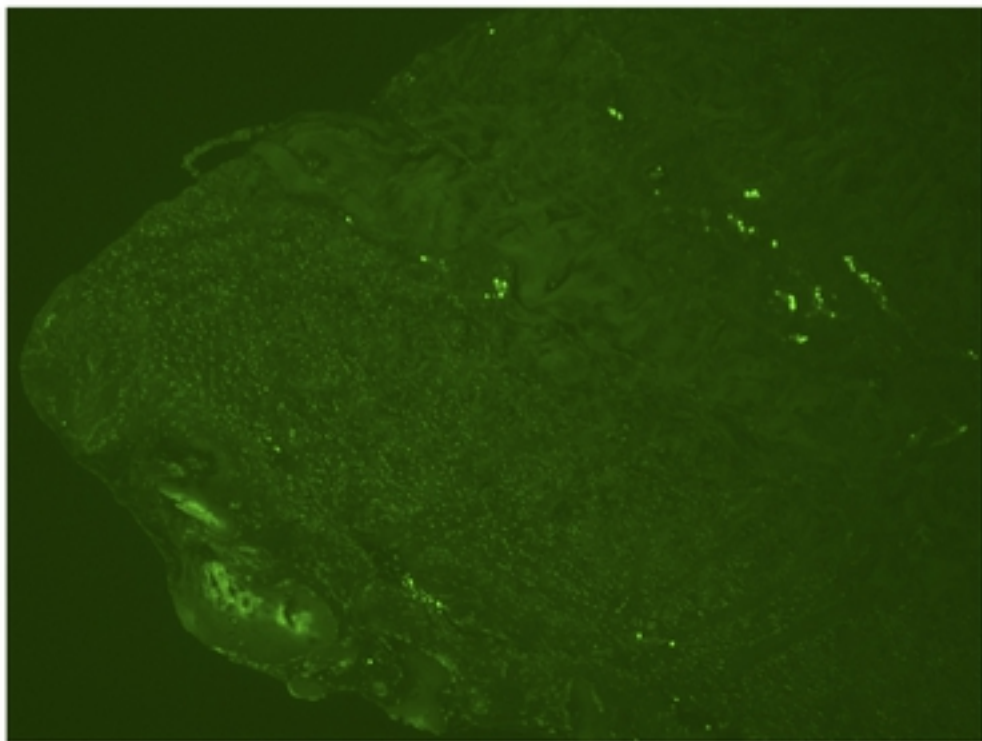


Figure5b



c

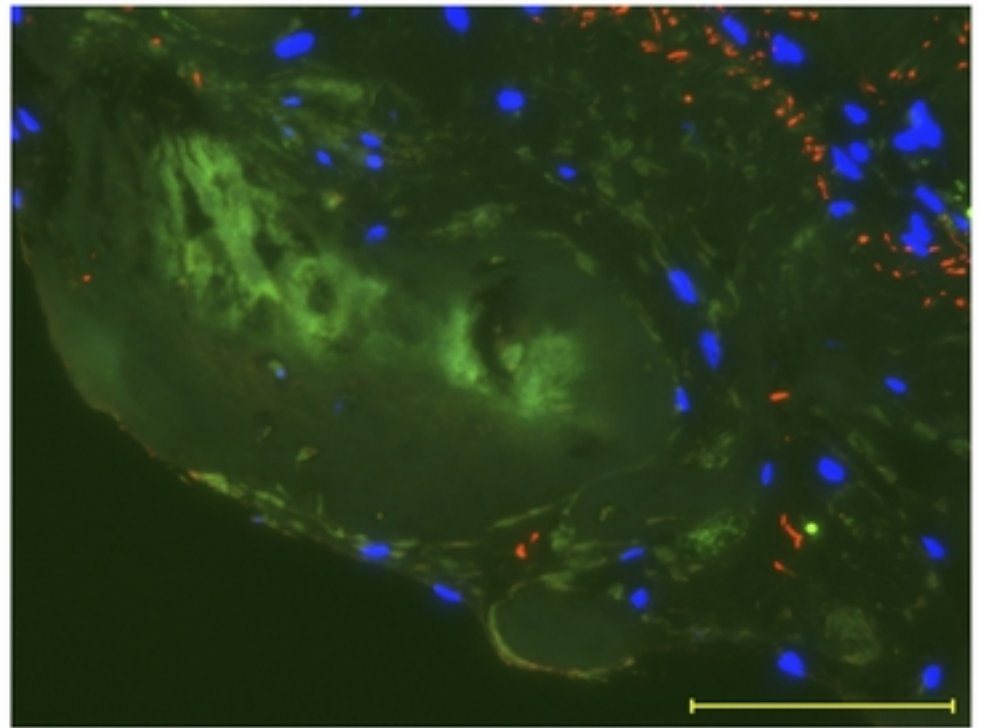
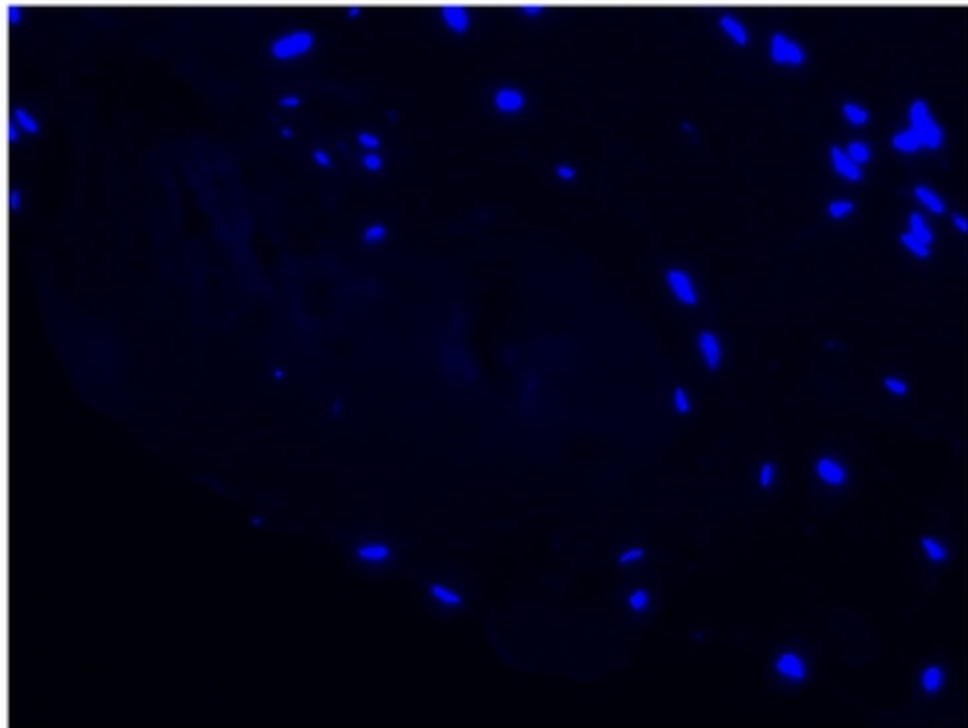
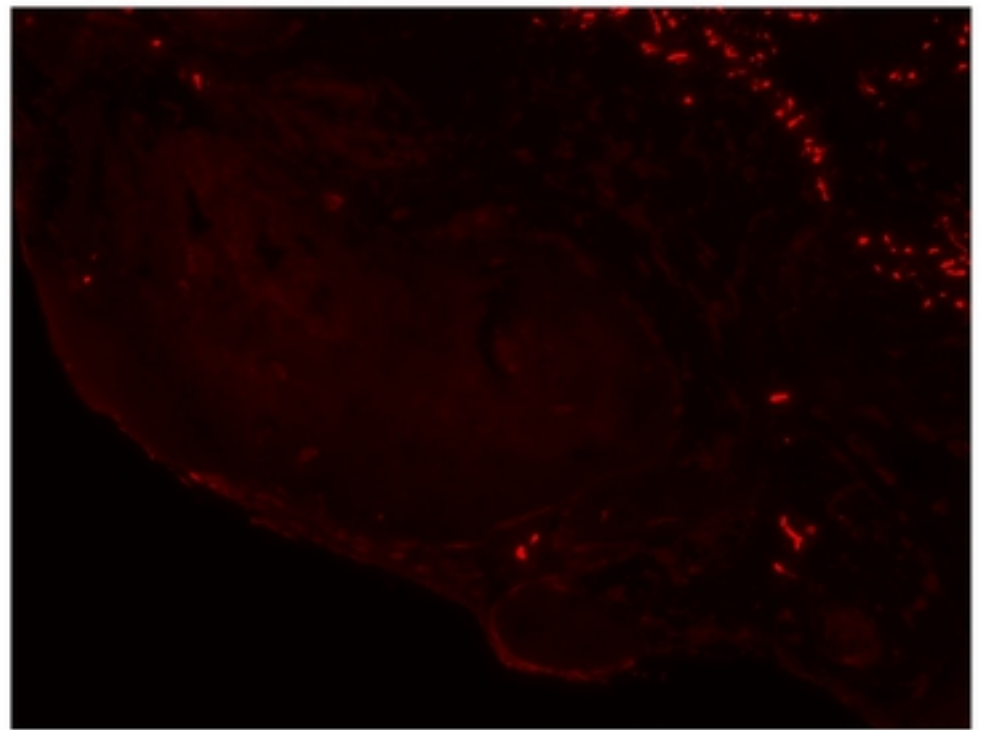
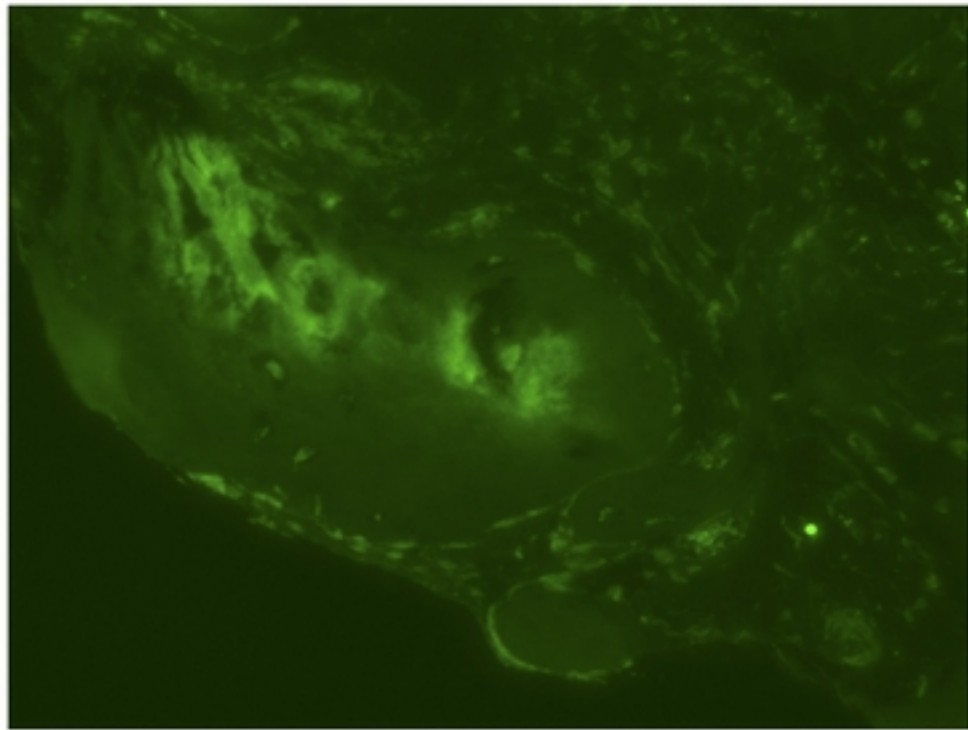


Figure5c

d

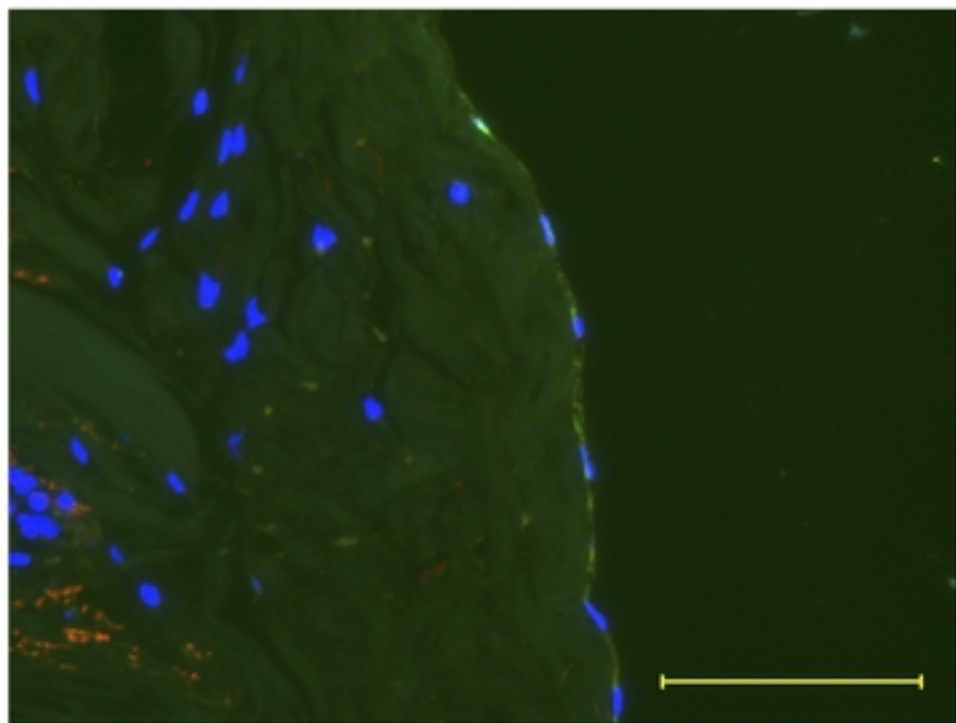
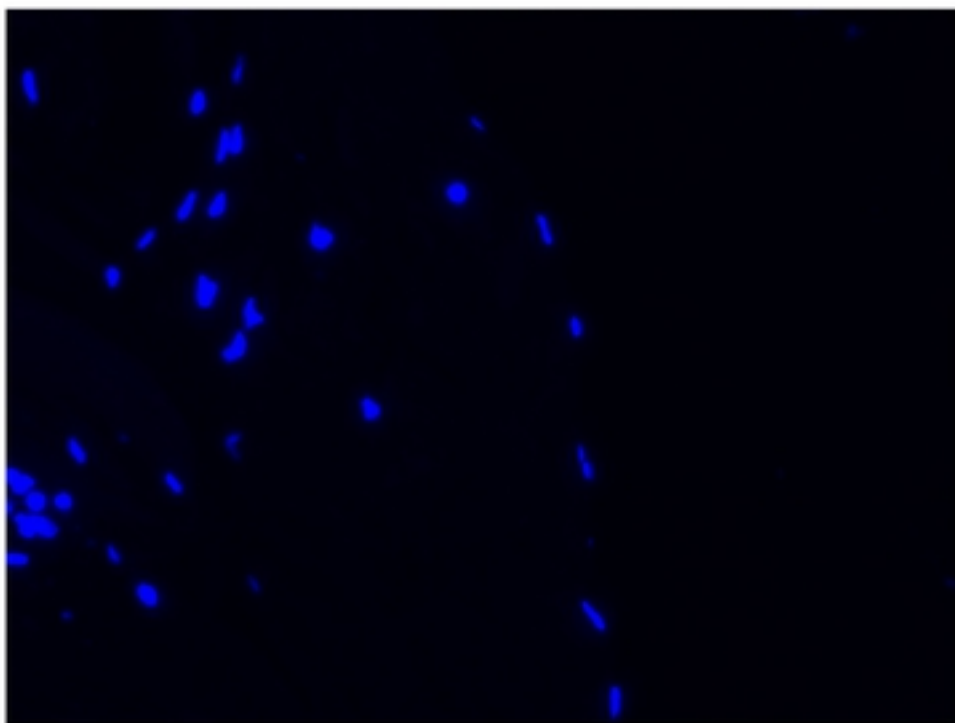
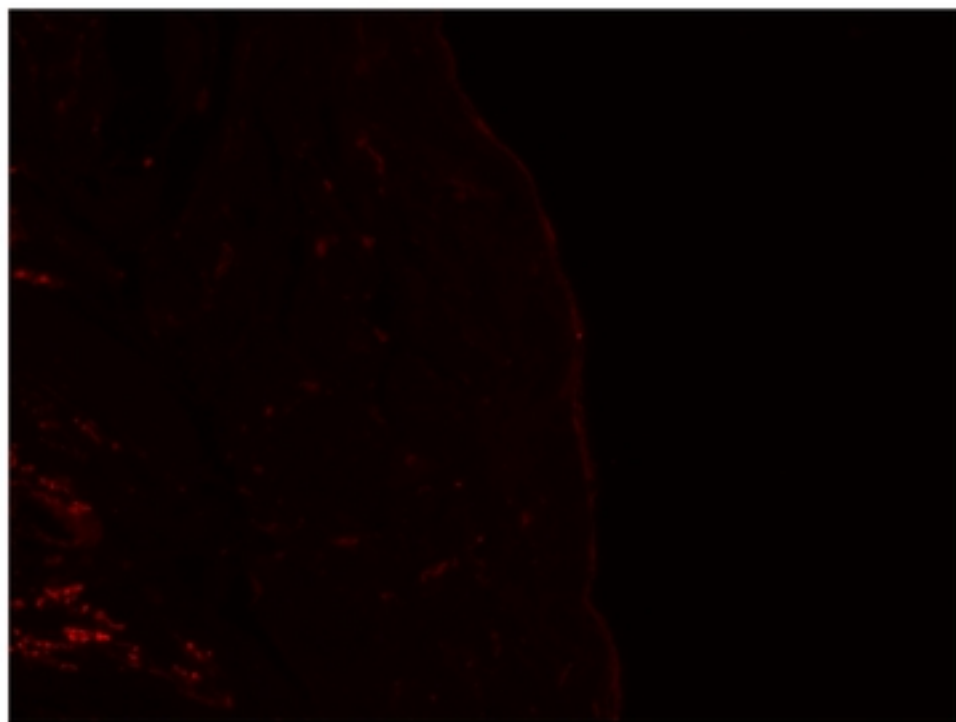
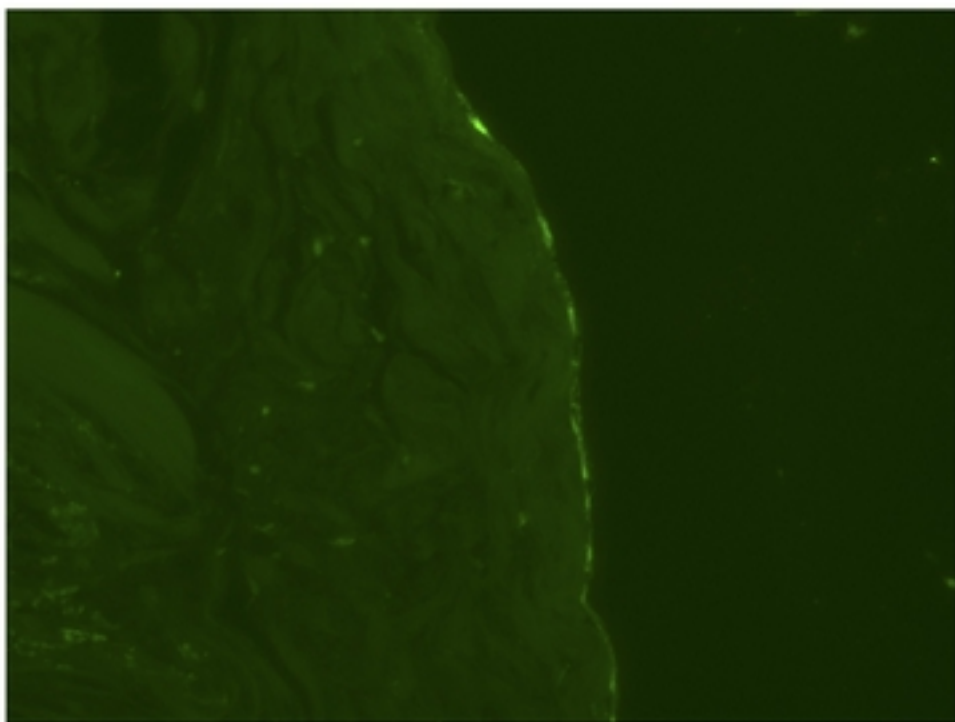


Figure5d

e

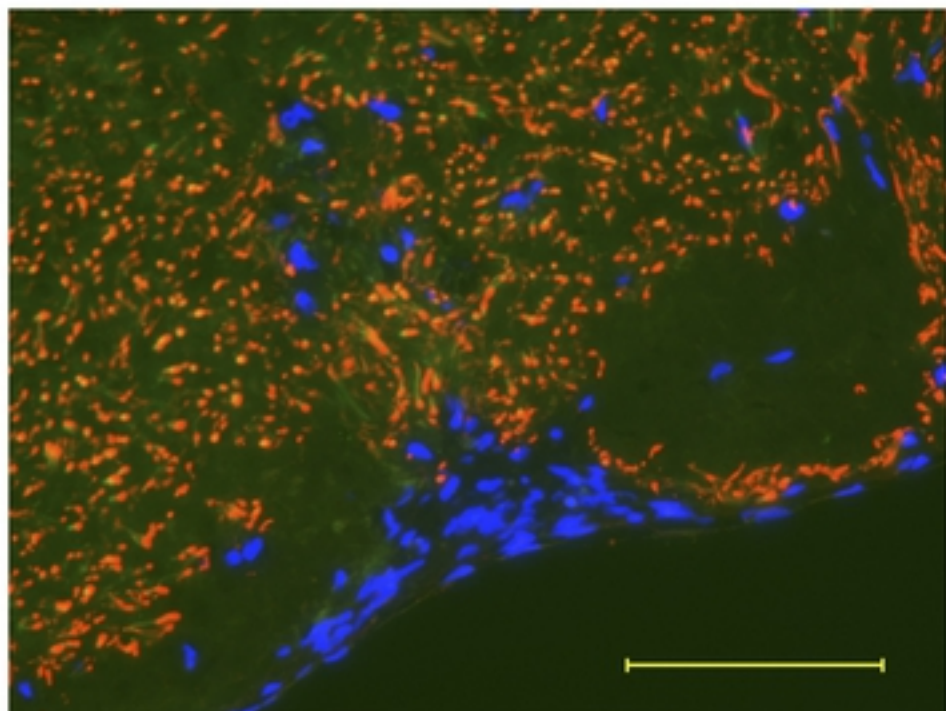
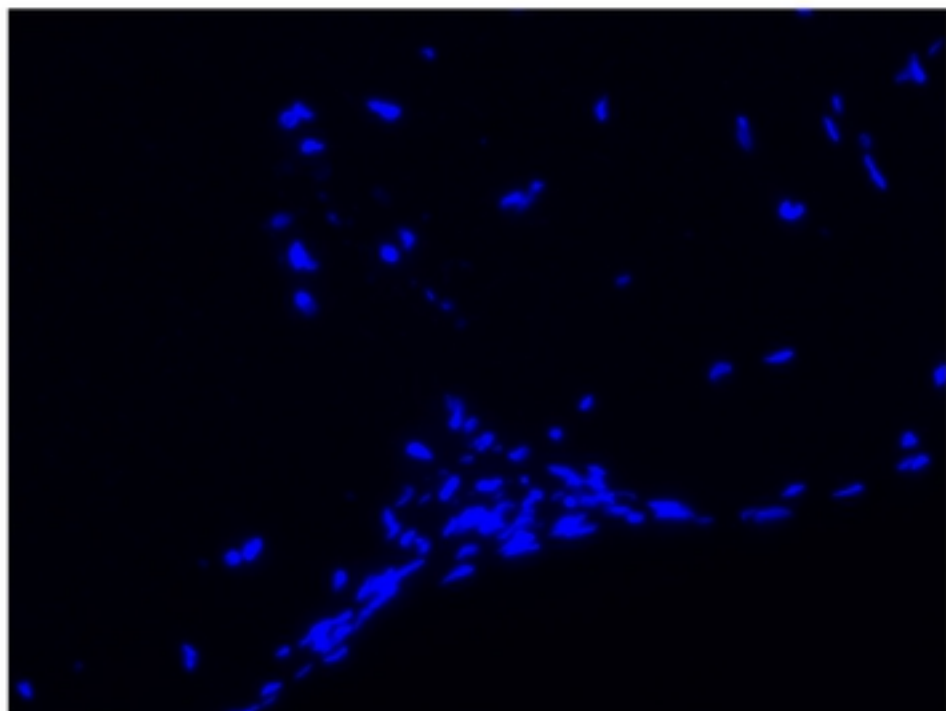
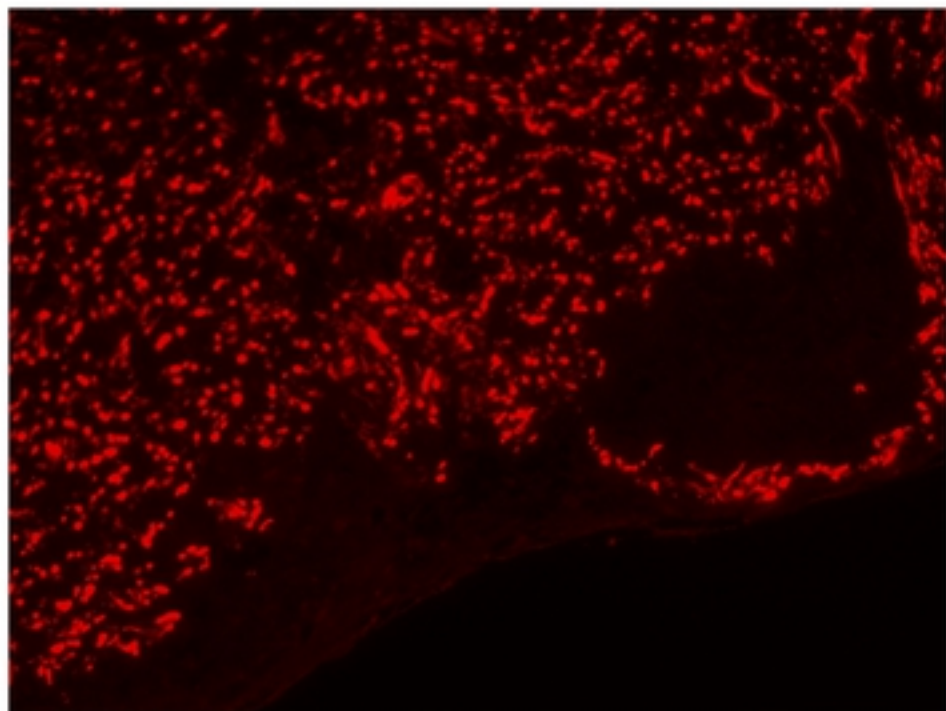
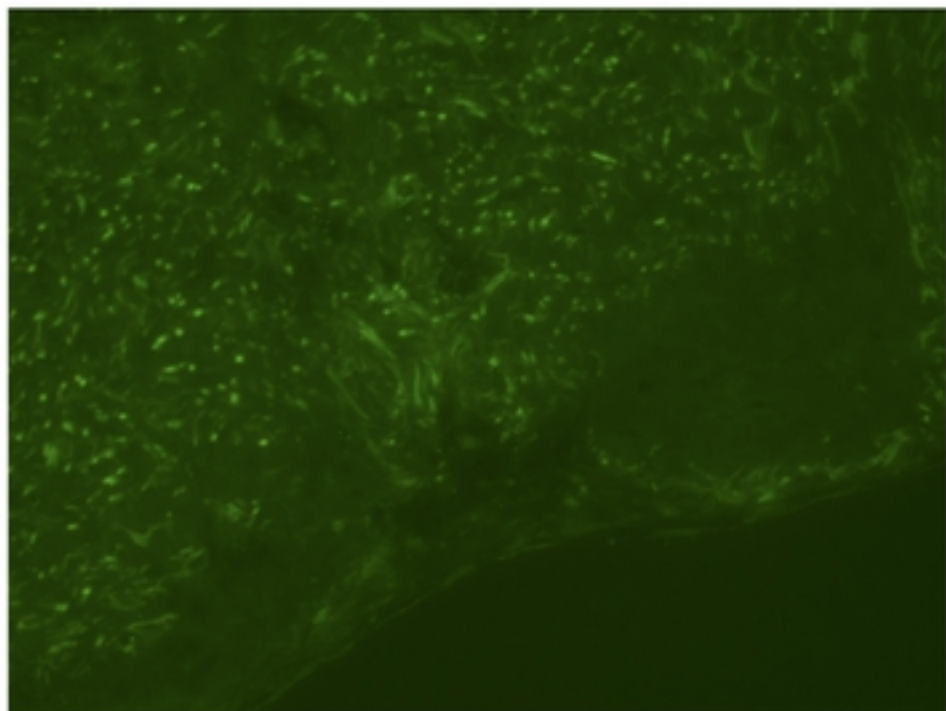


Figure5e

f

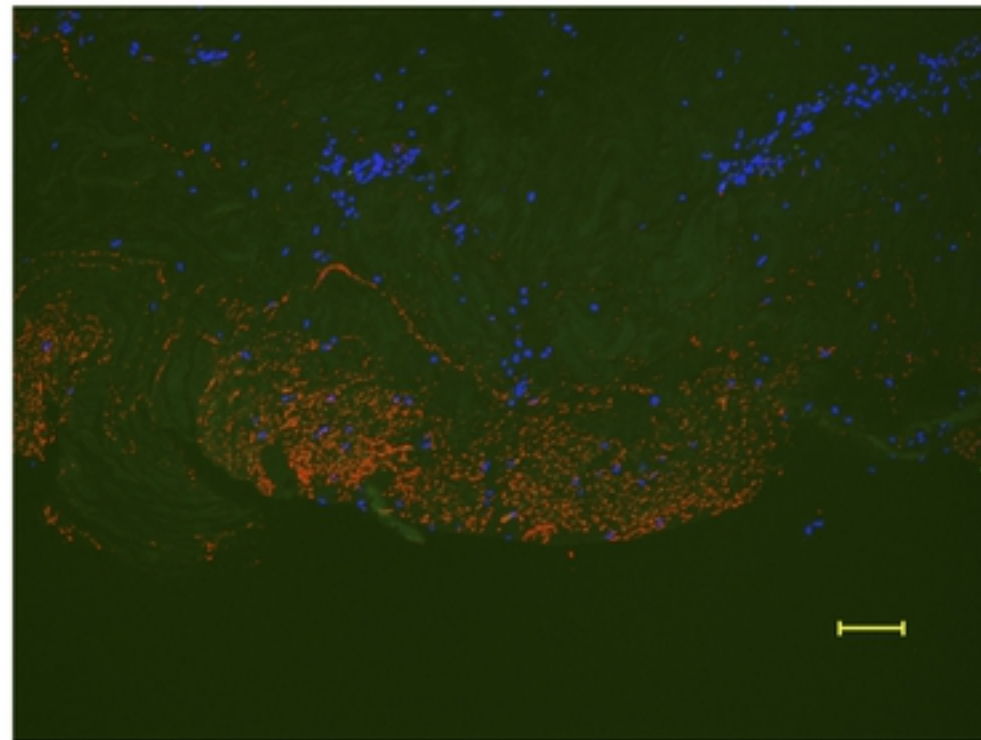
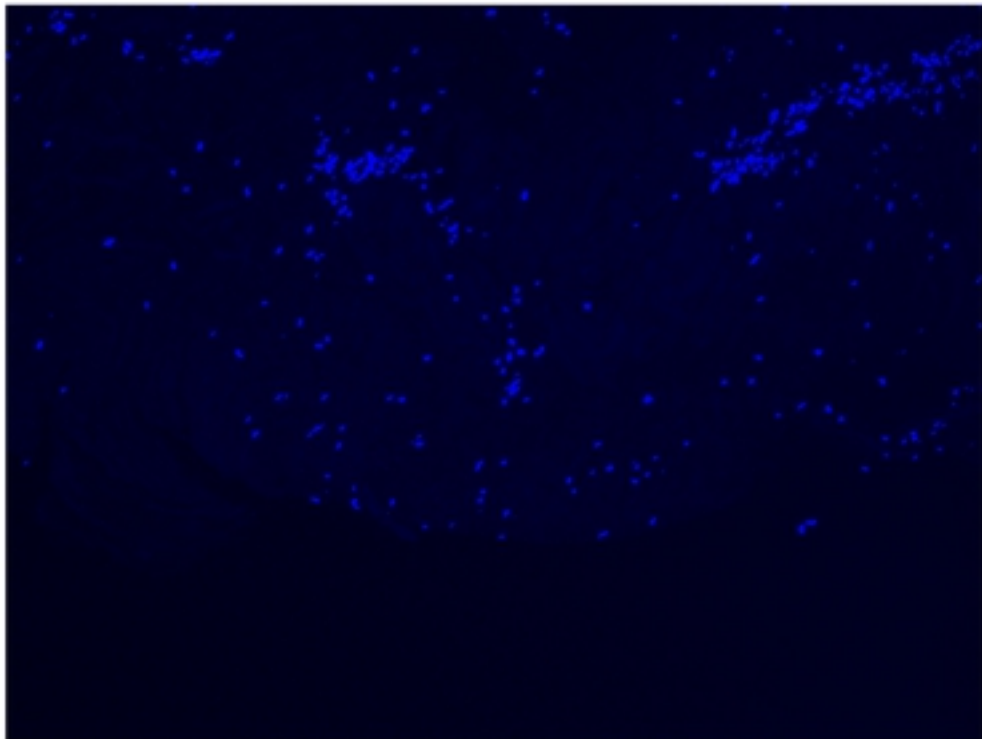
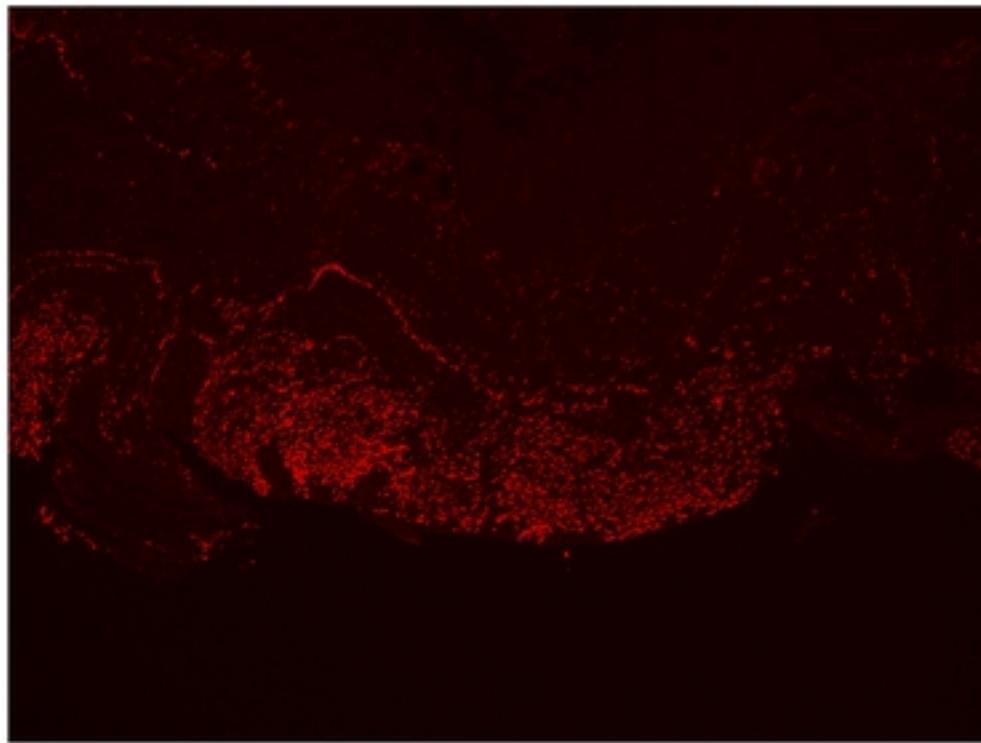
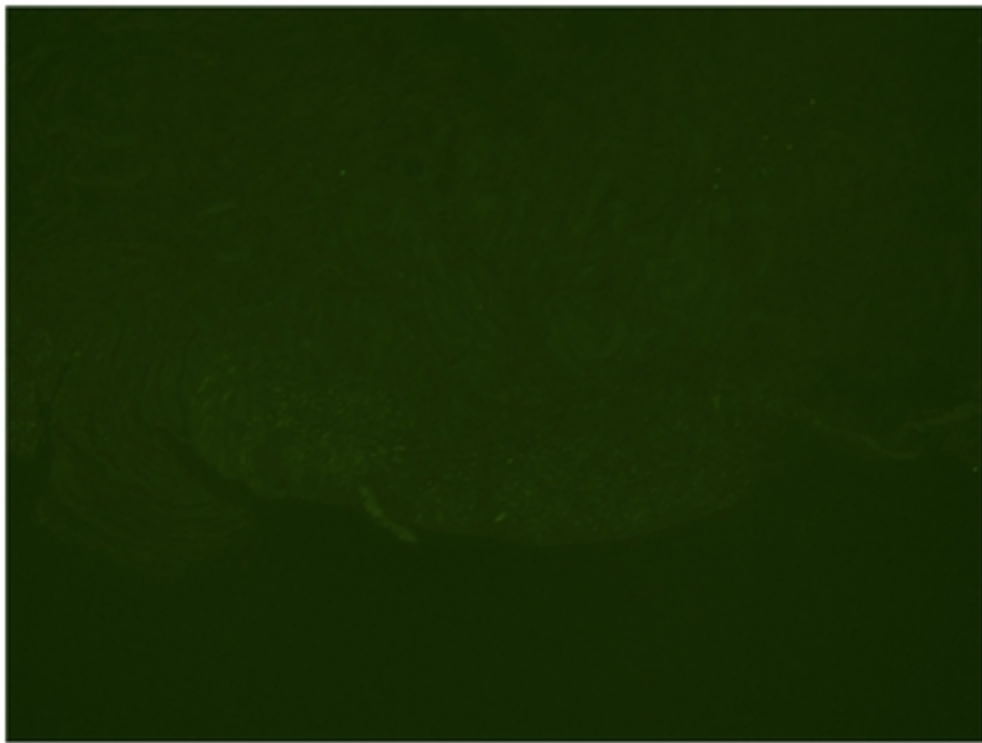


Figure5f

a

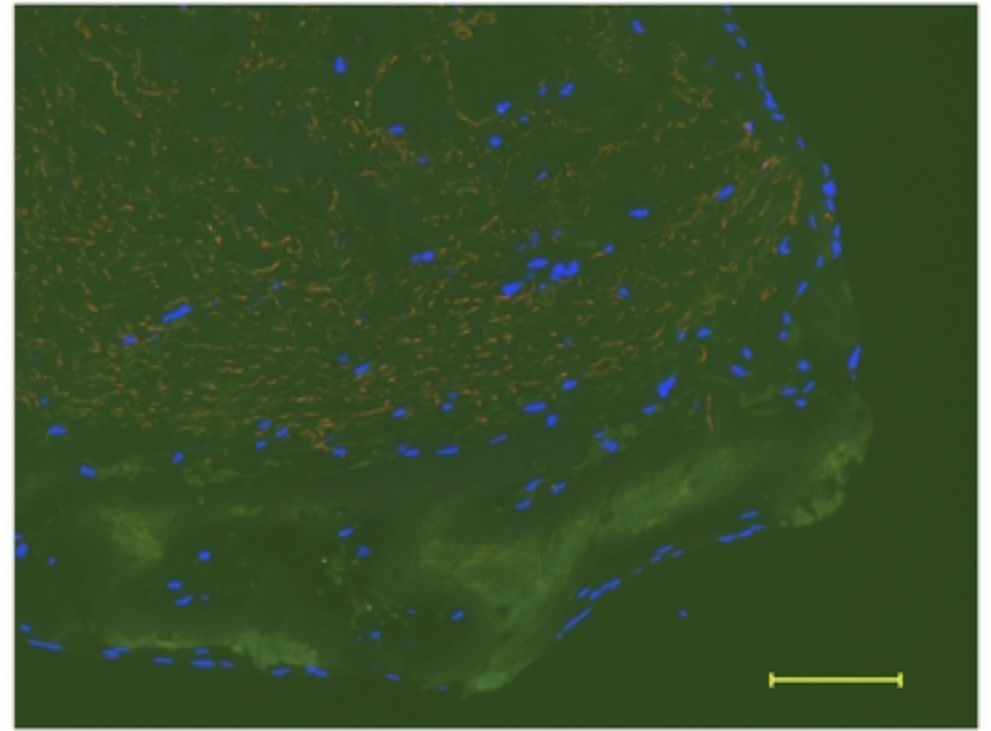
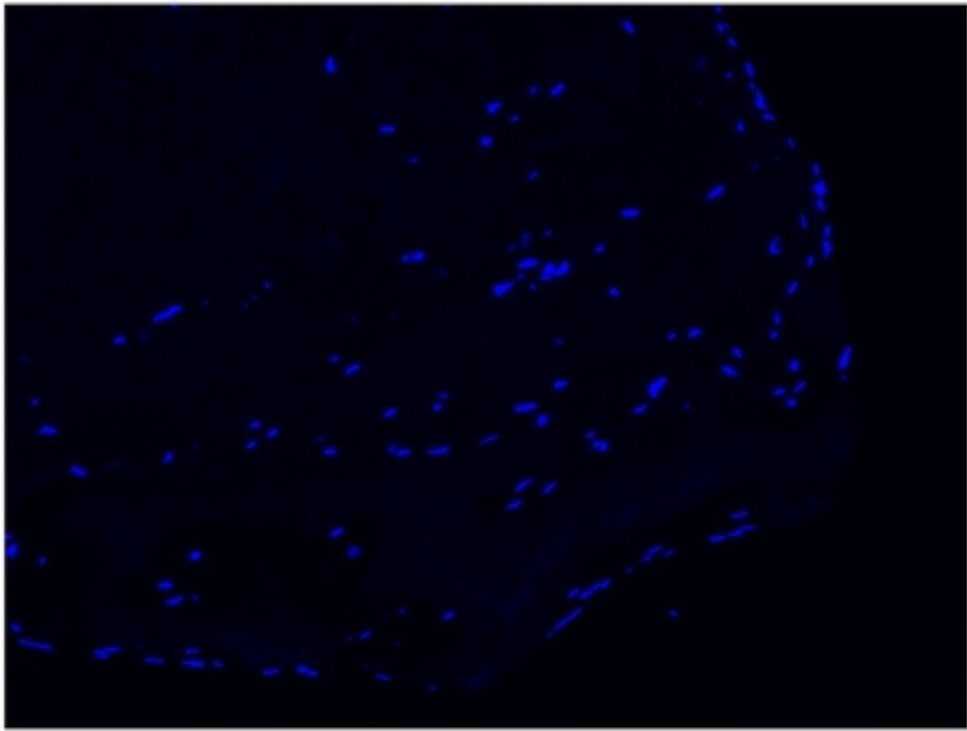
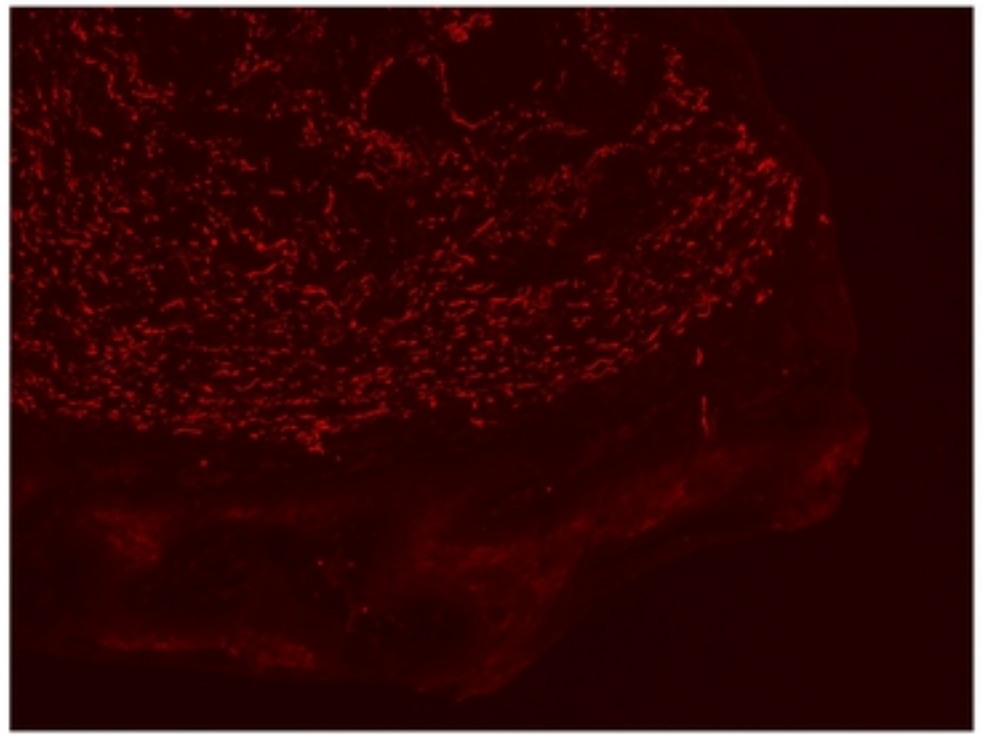
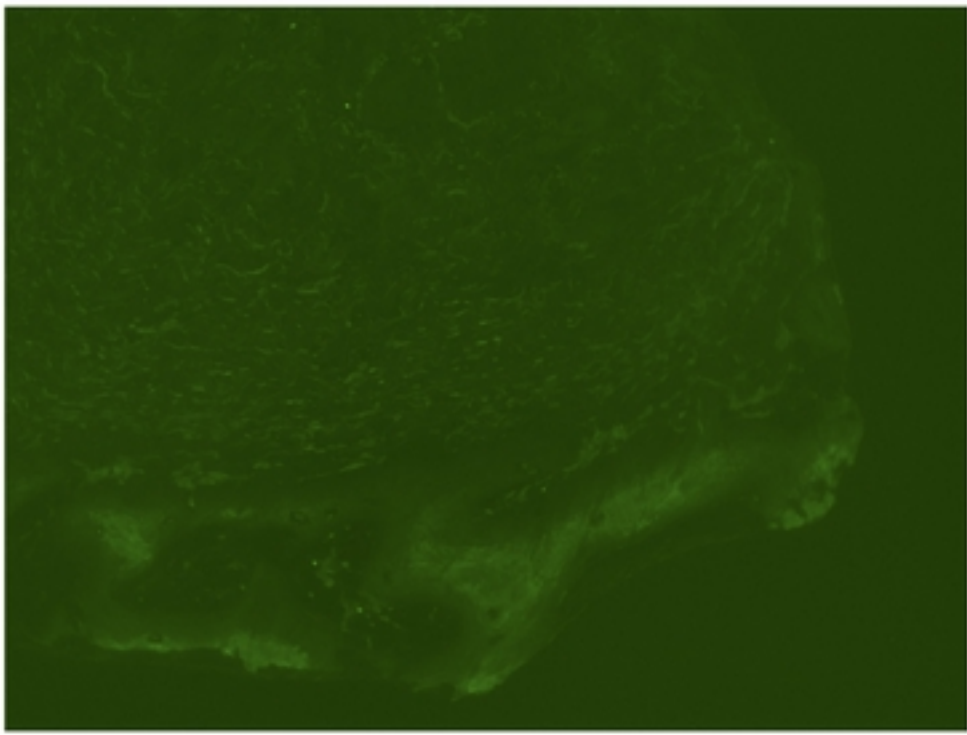


Figure6a

b

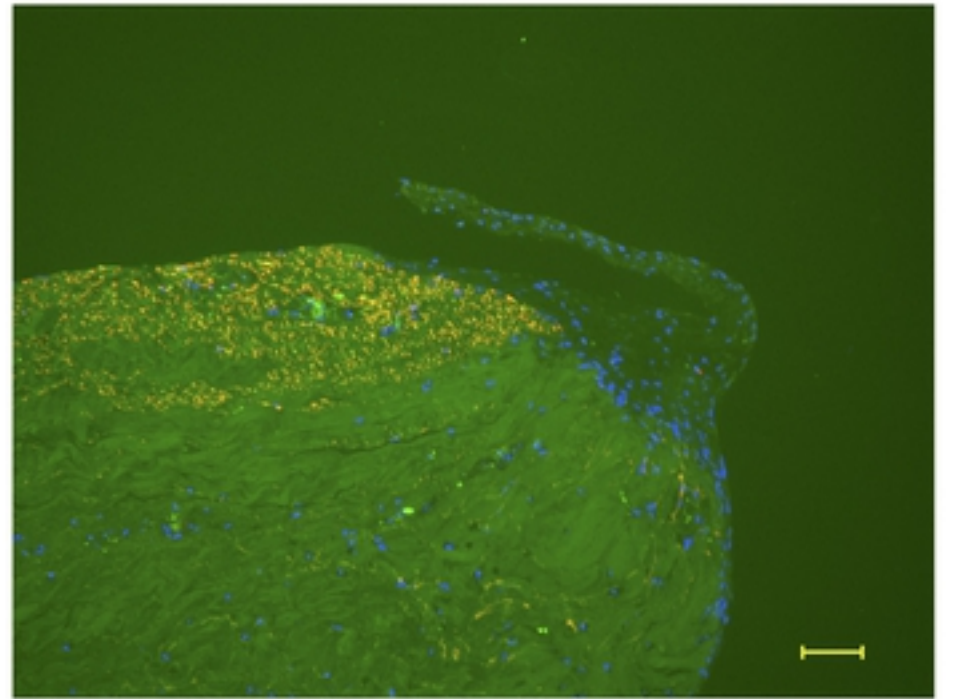
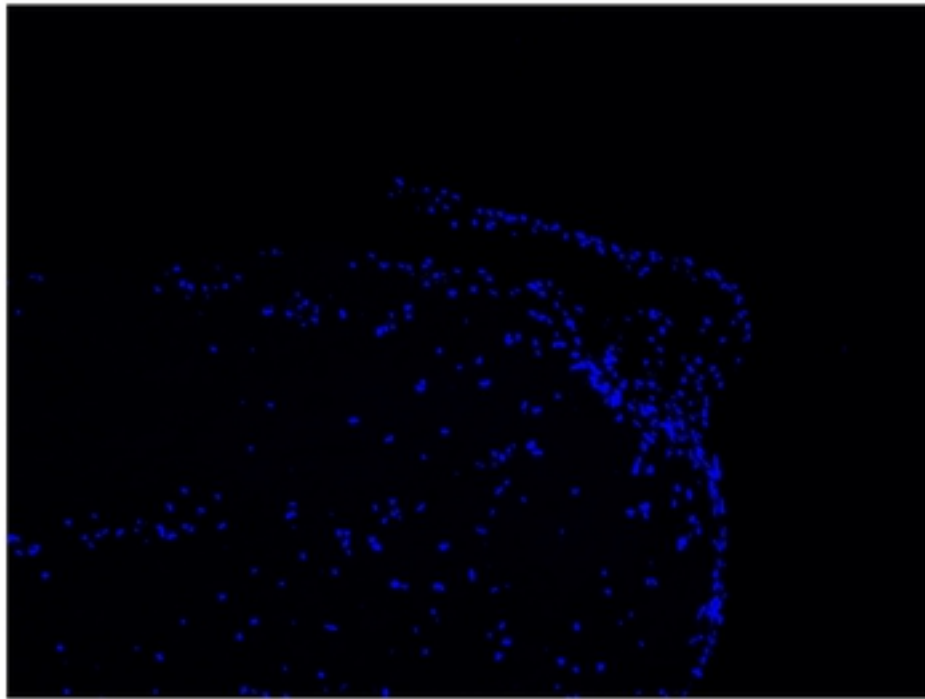
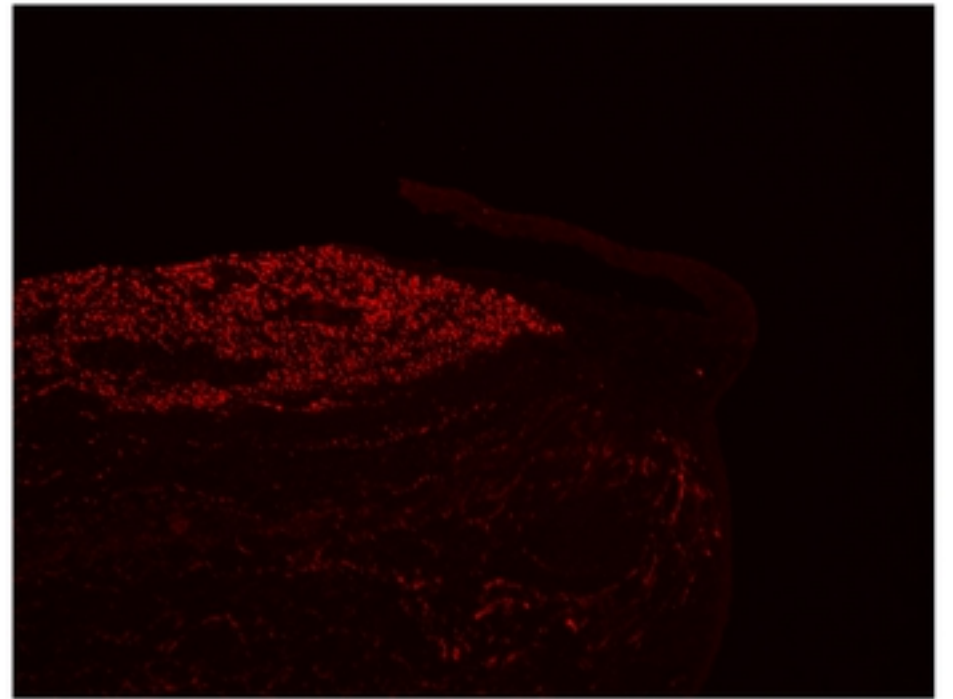
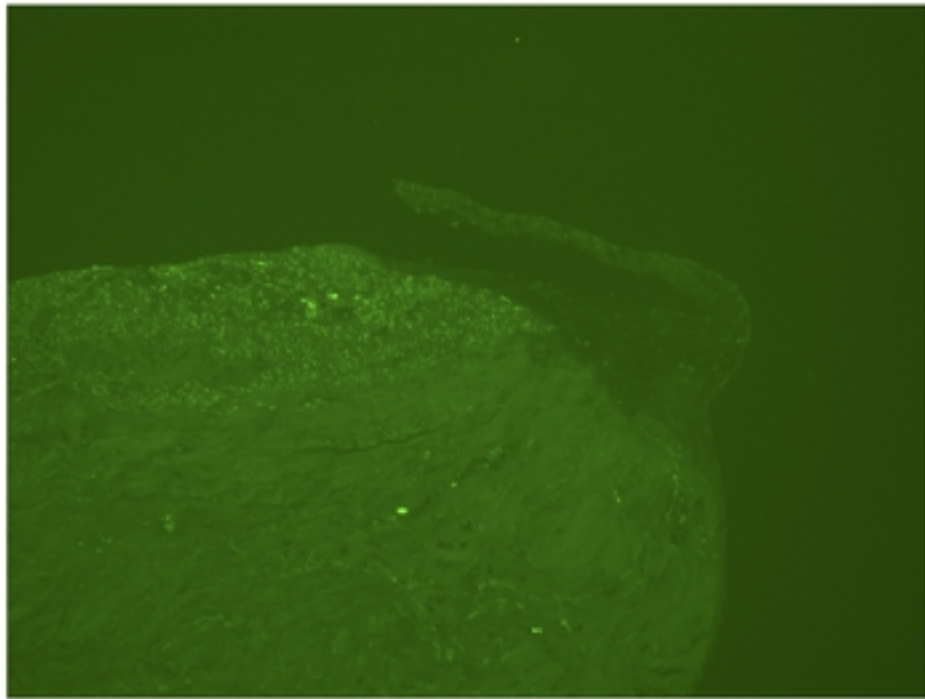


Figure6b

c

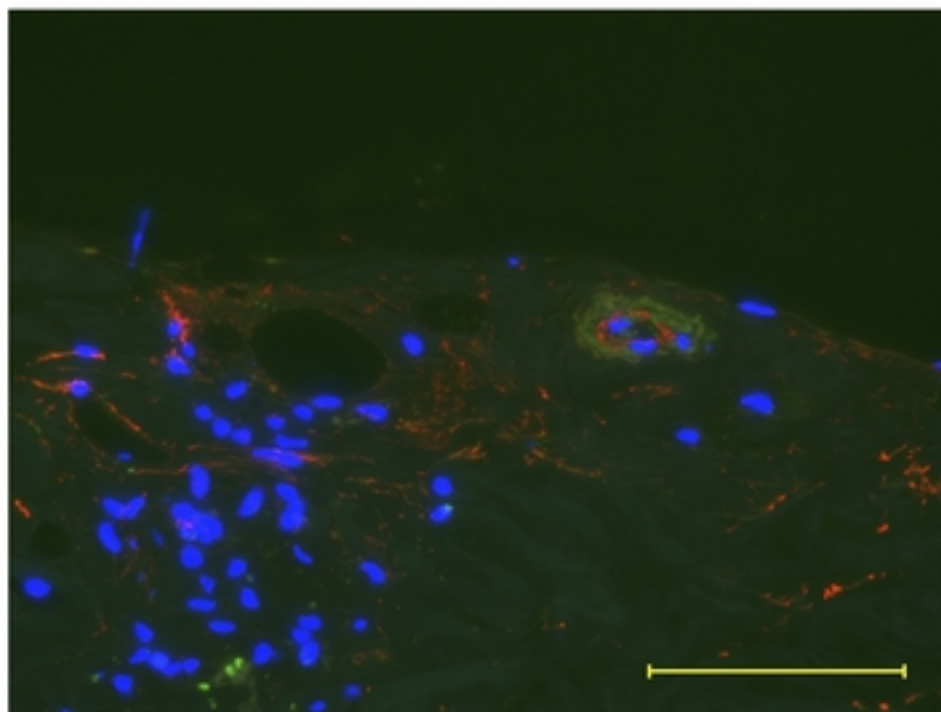
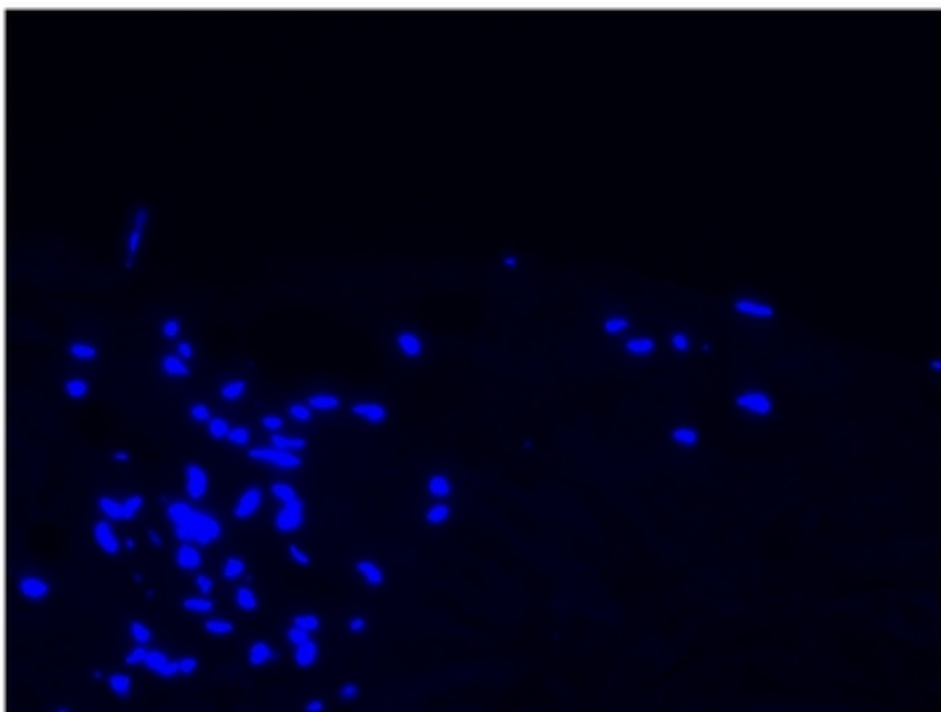
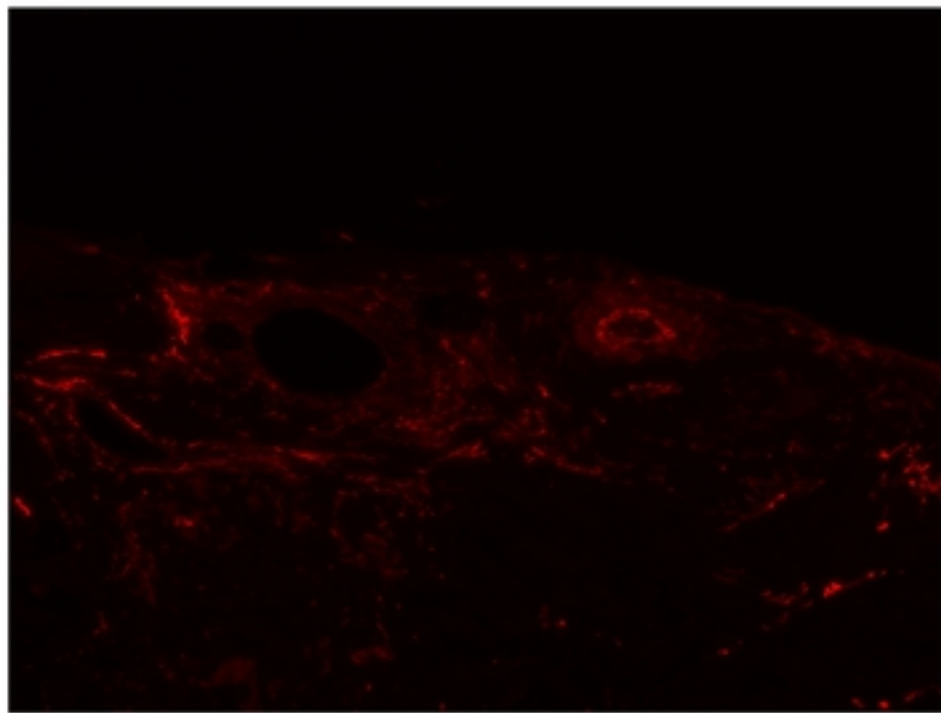
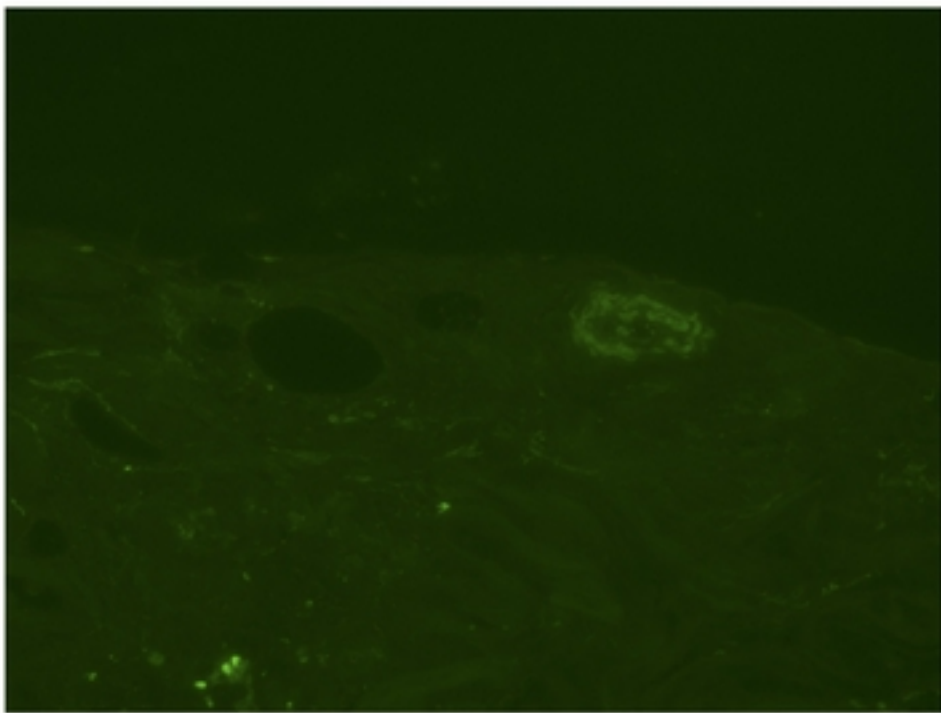


Figure6c

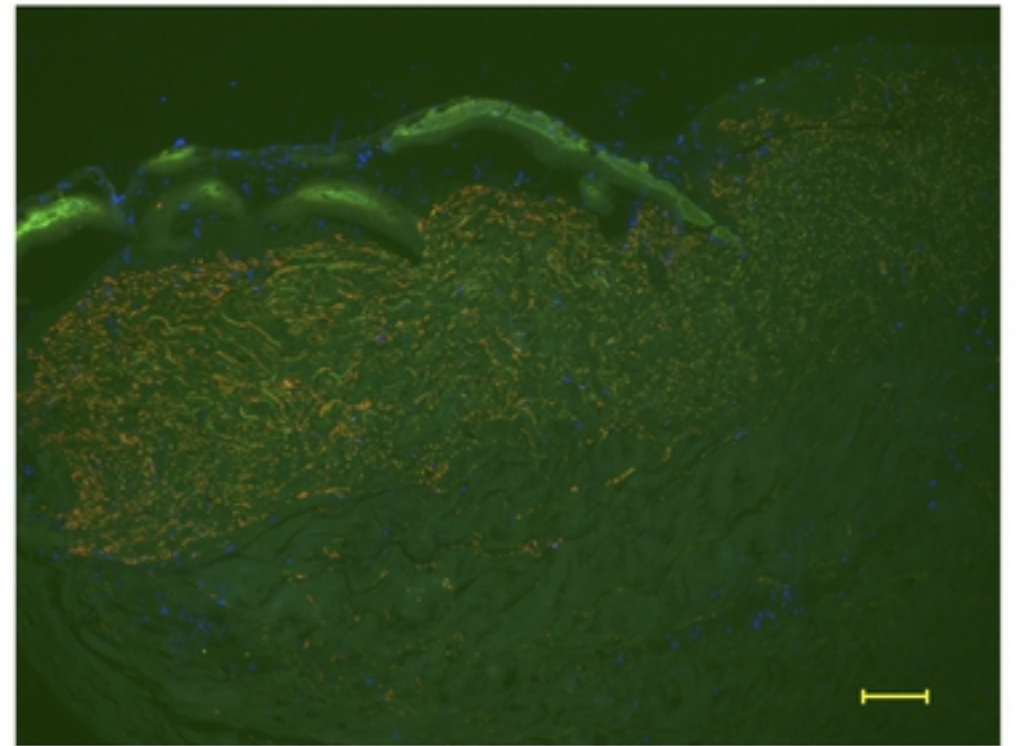
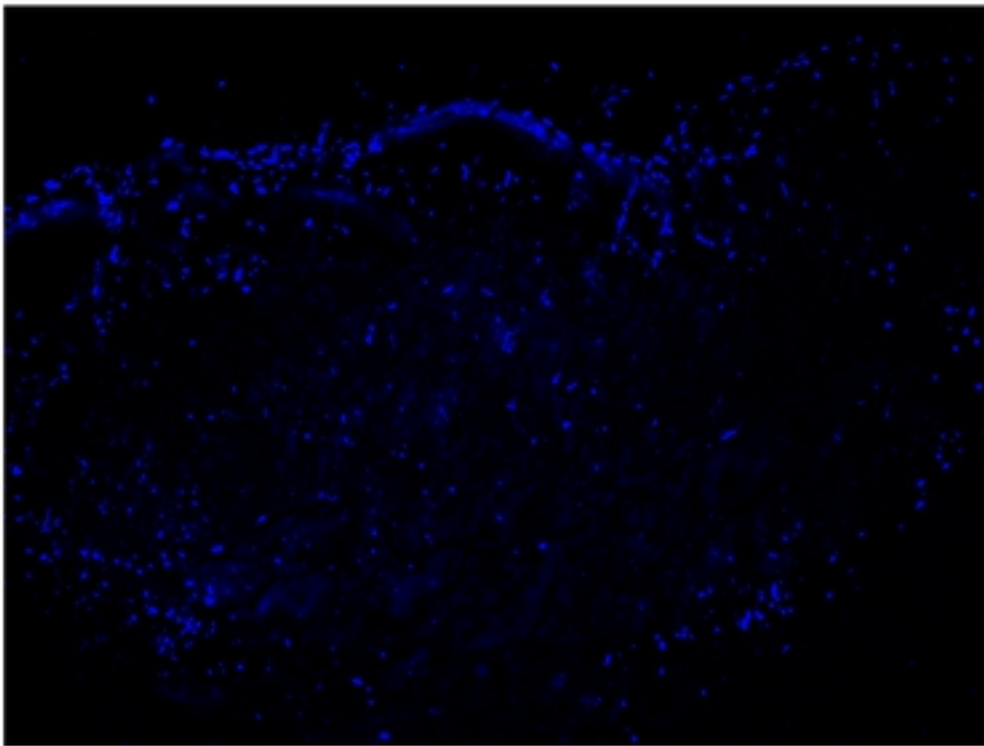
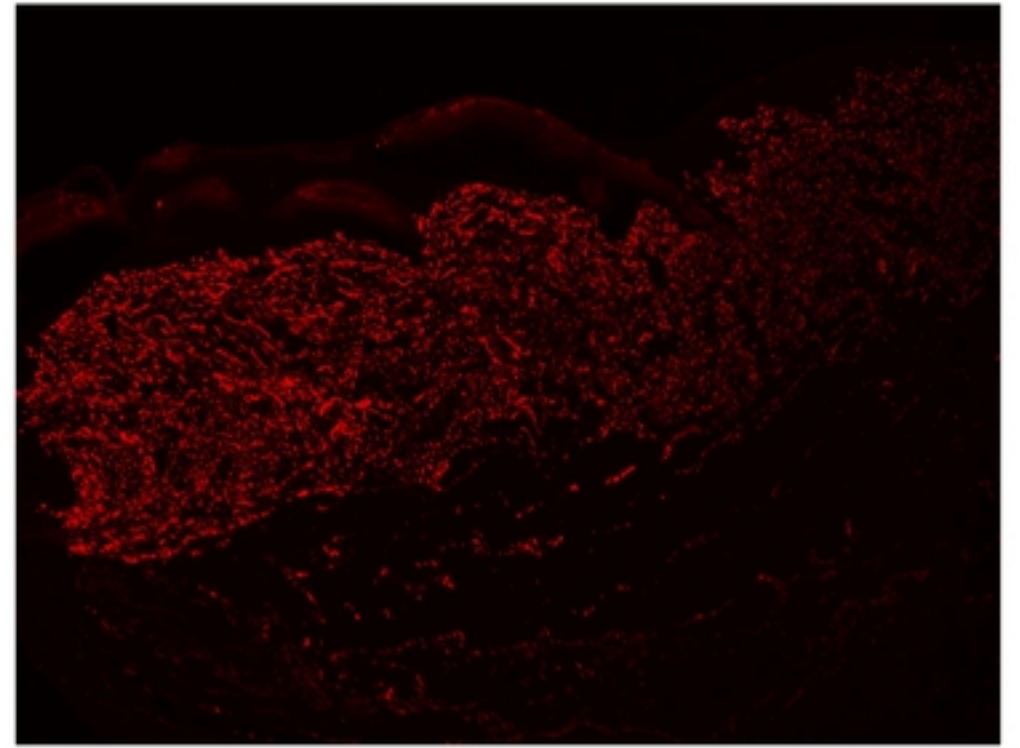
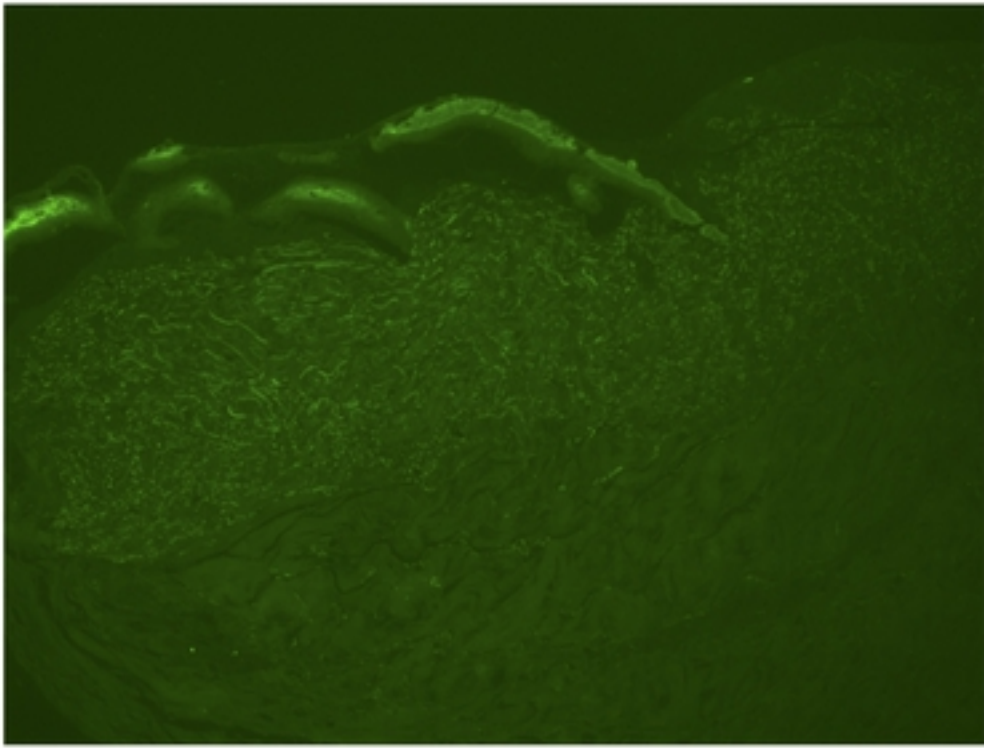


Figure7



a

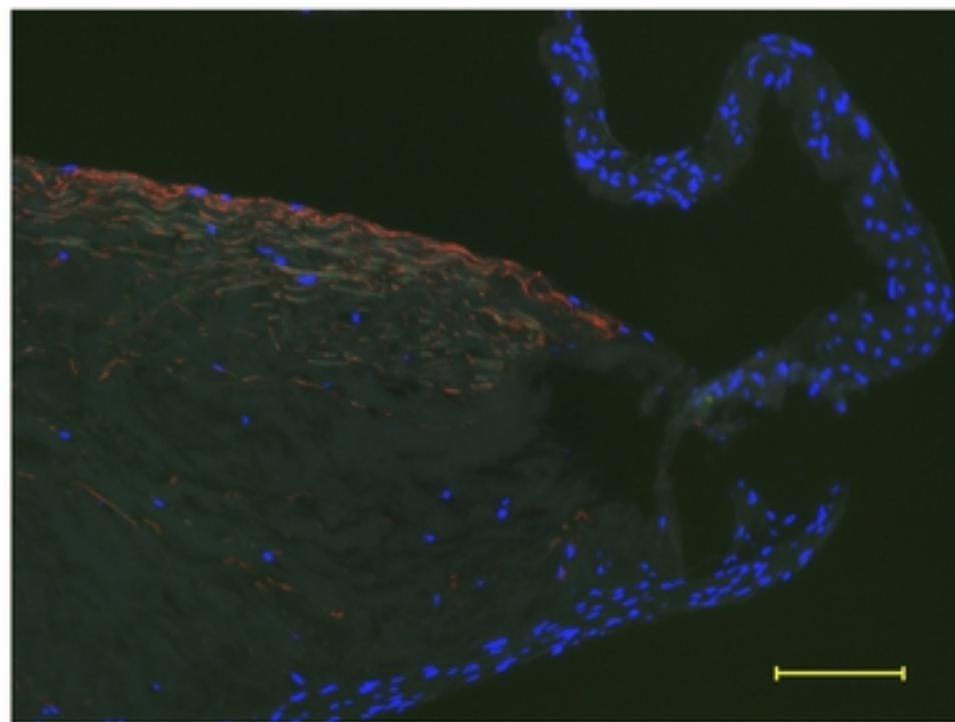
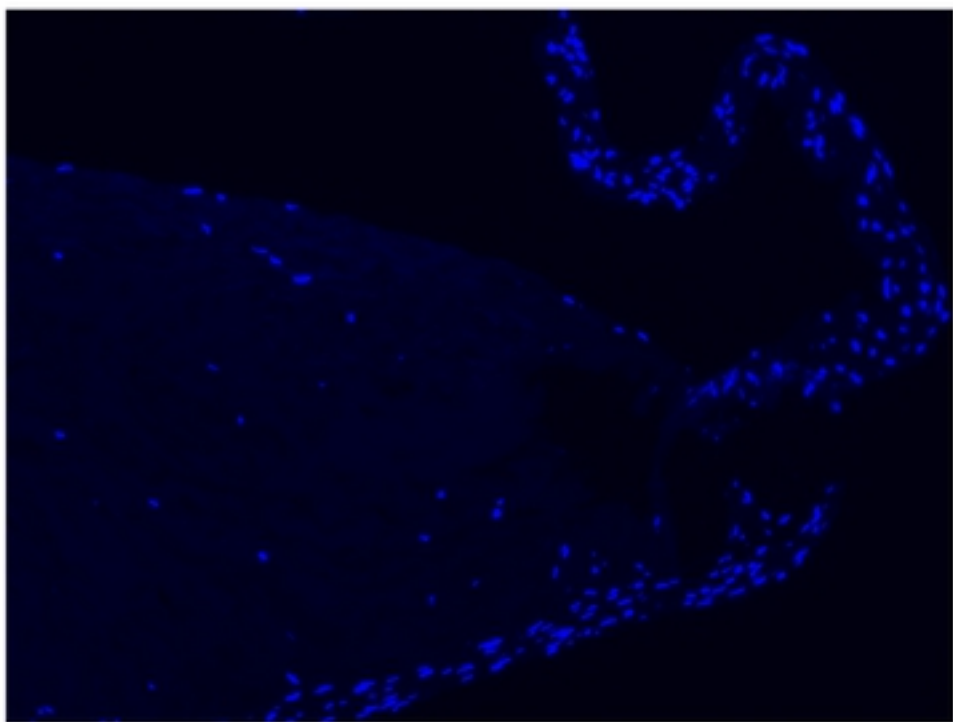
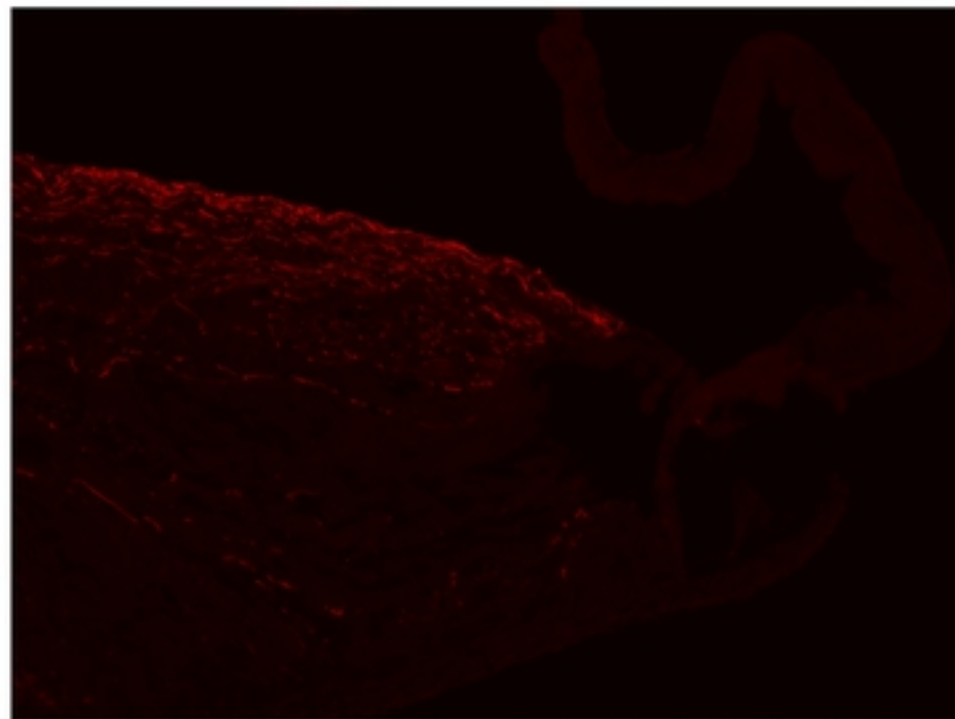
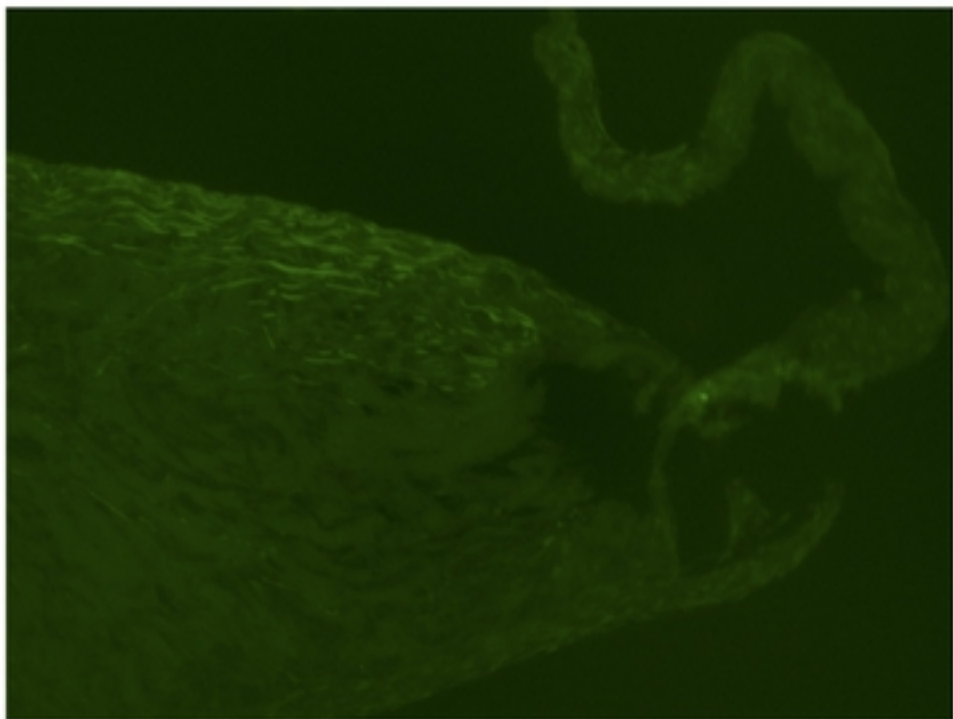


Figure8a

b

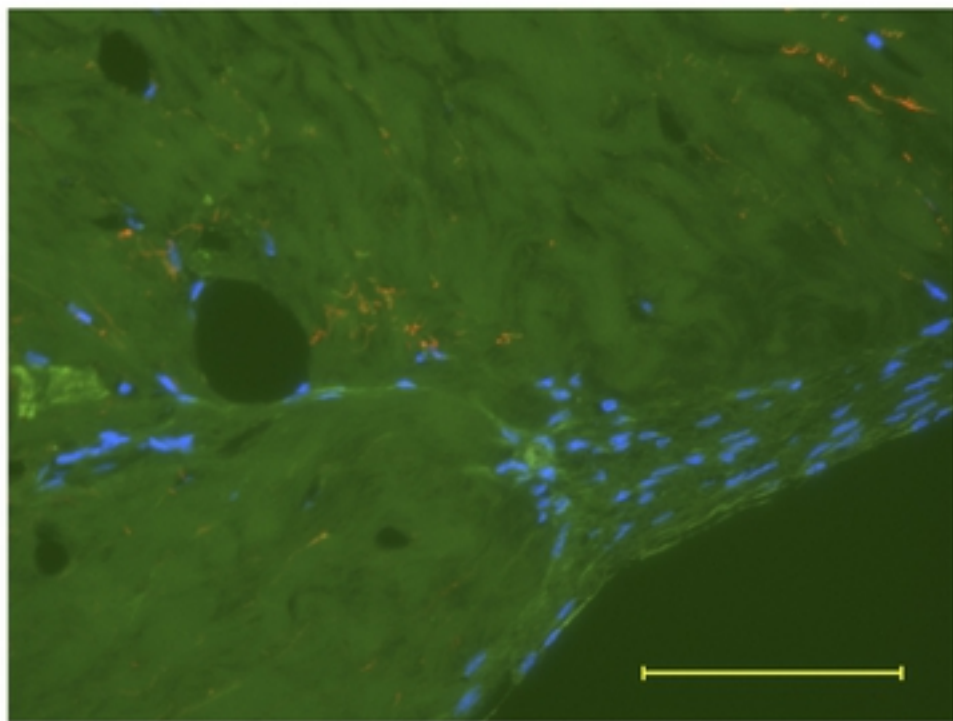
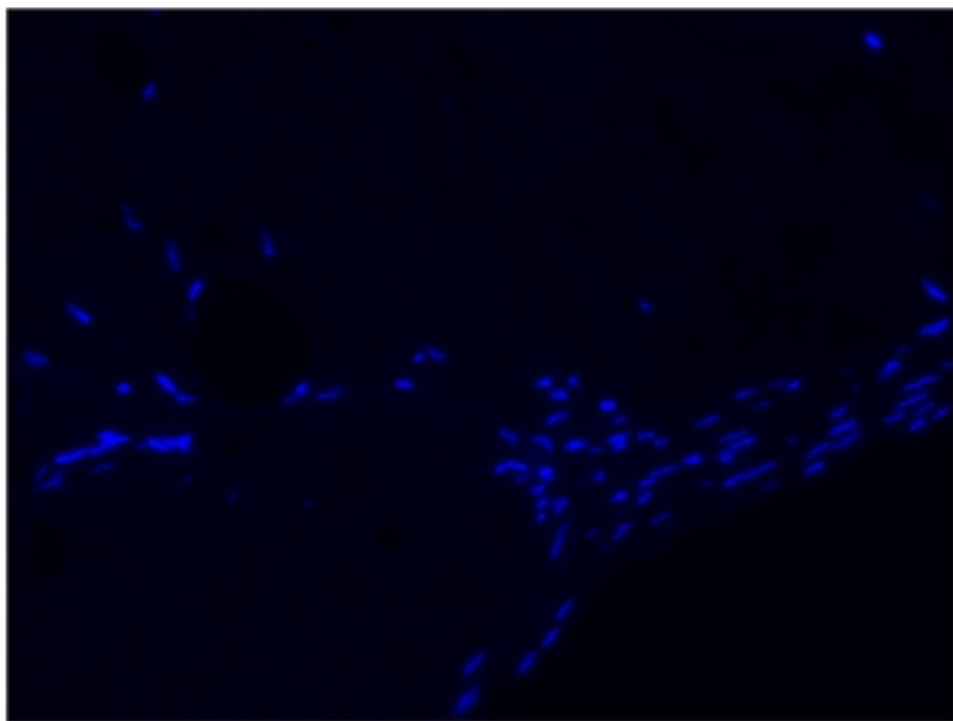
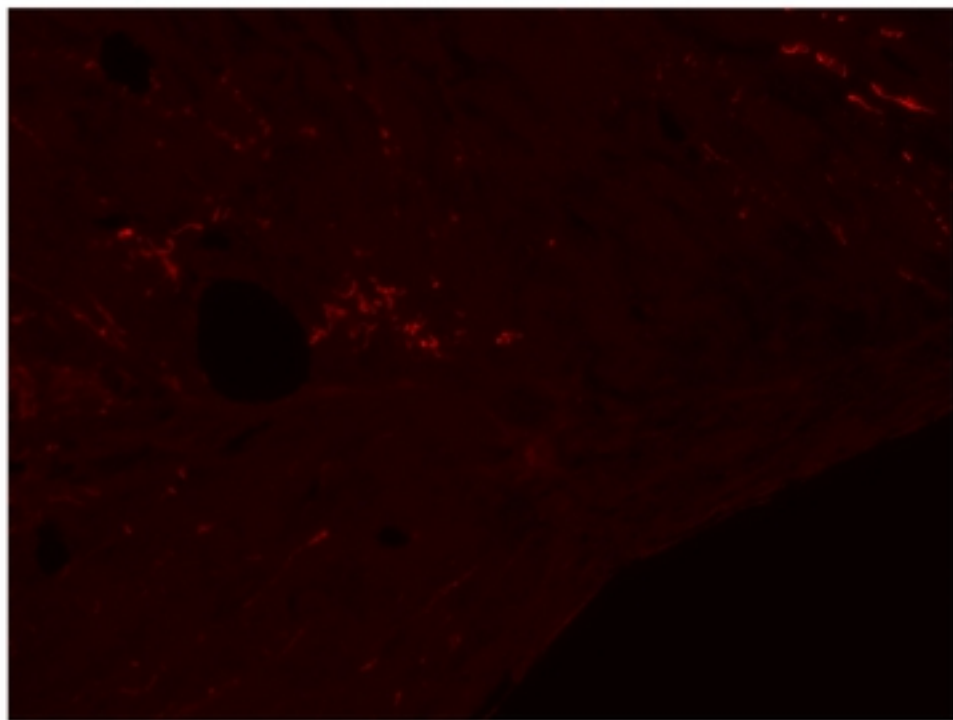
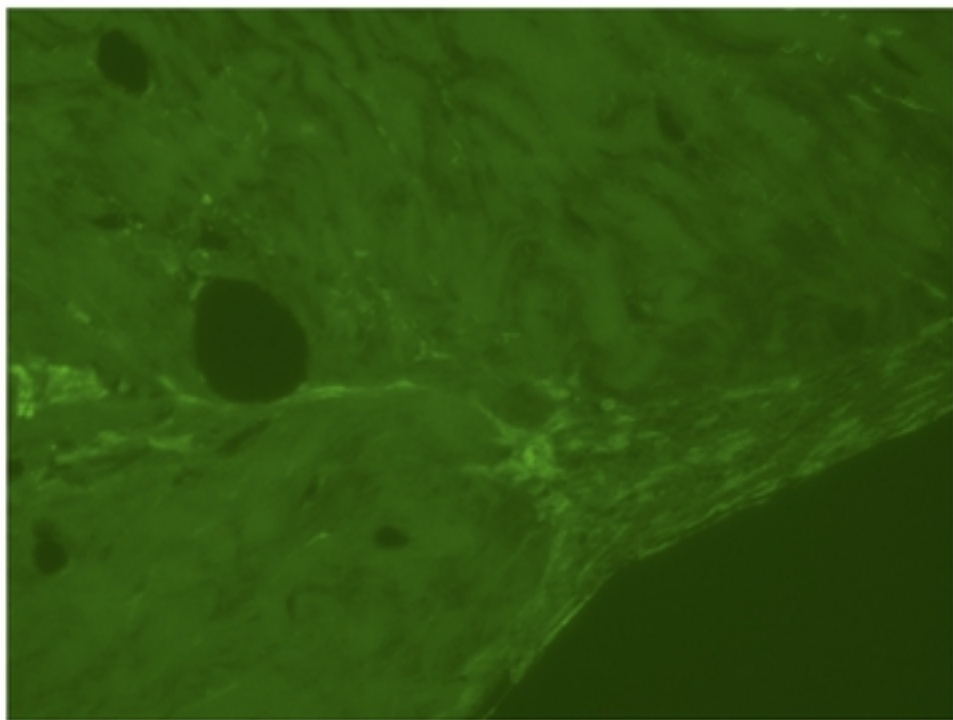


Figure8b

c

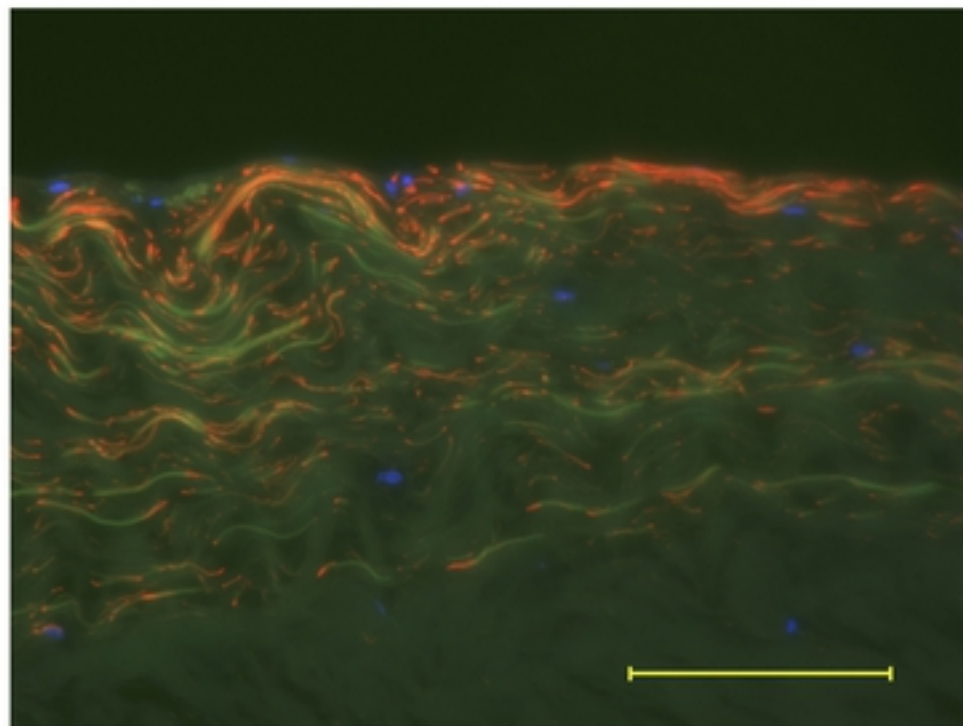
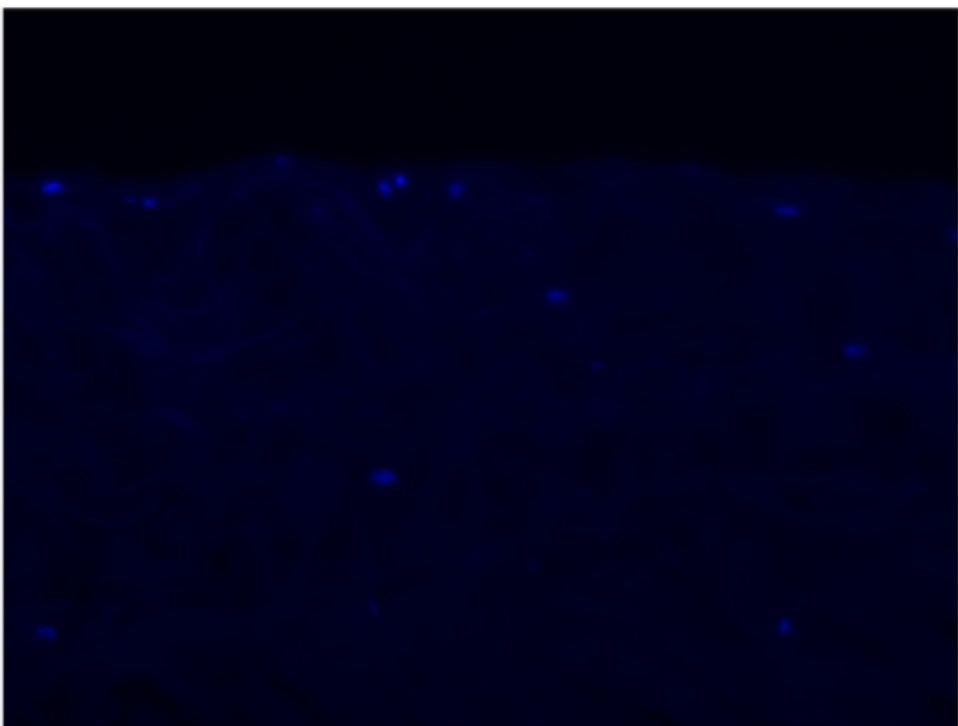
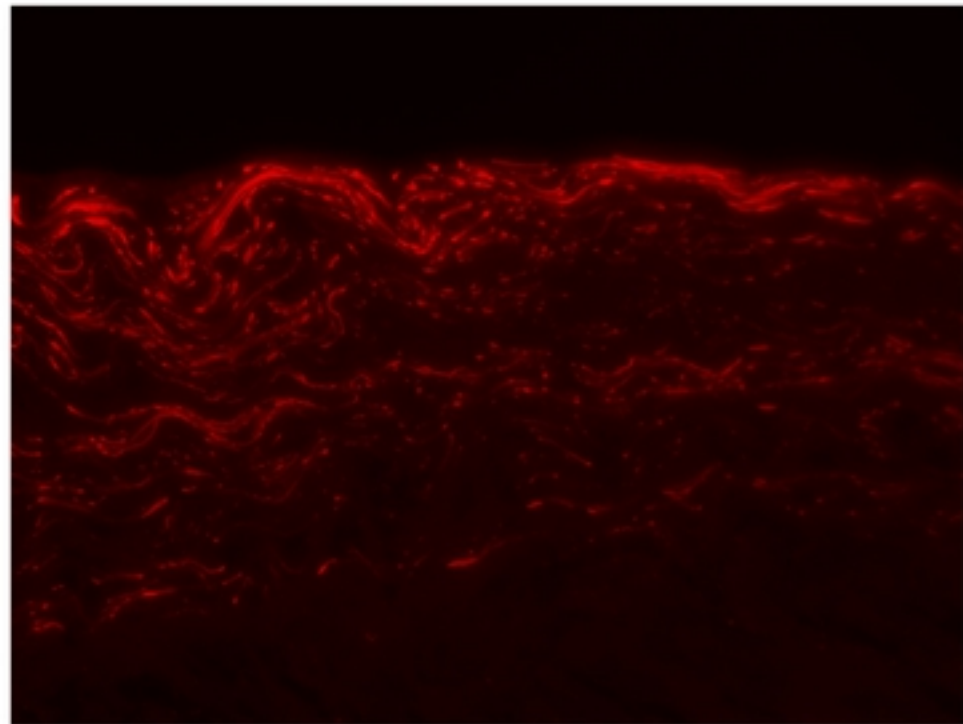
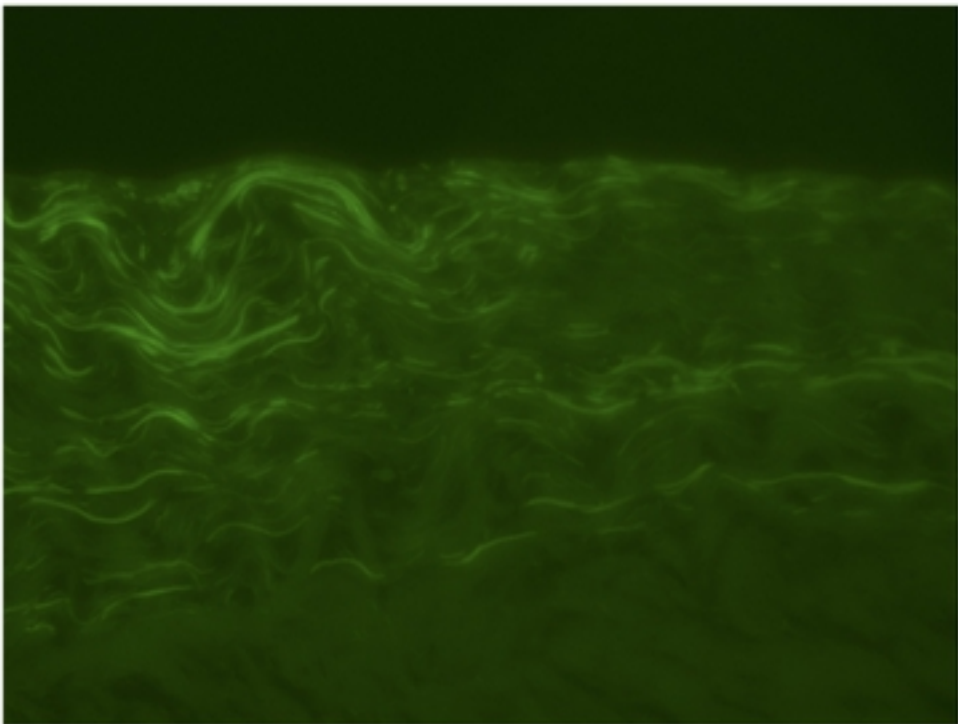


Figure8c

d

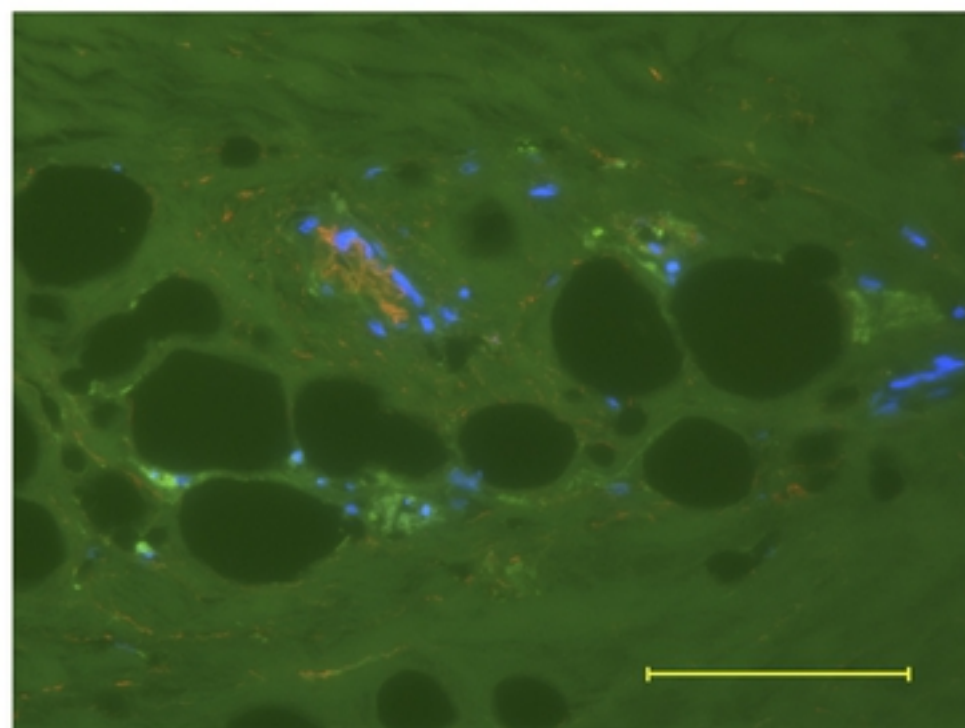
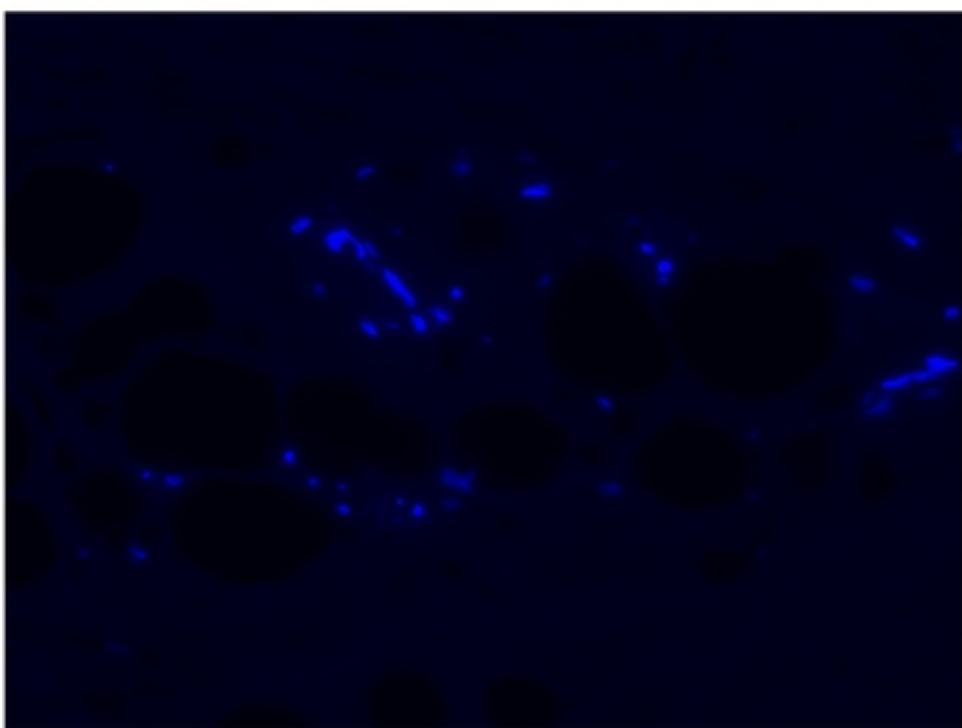
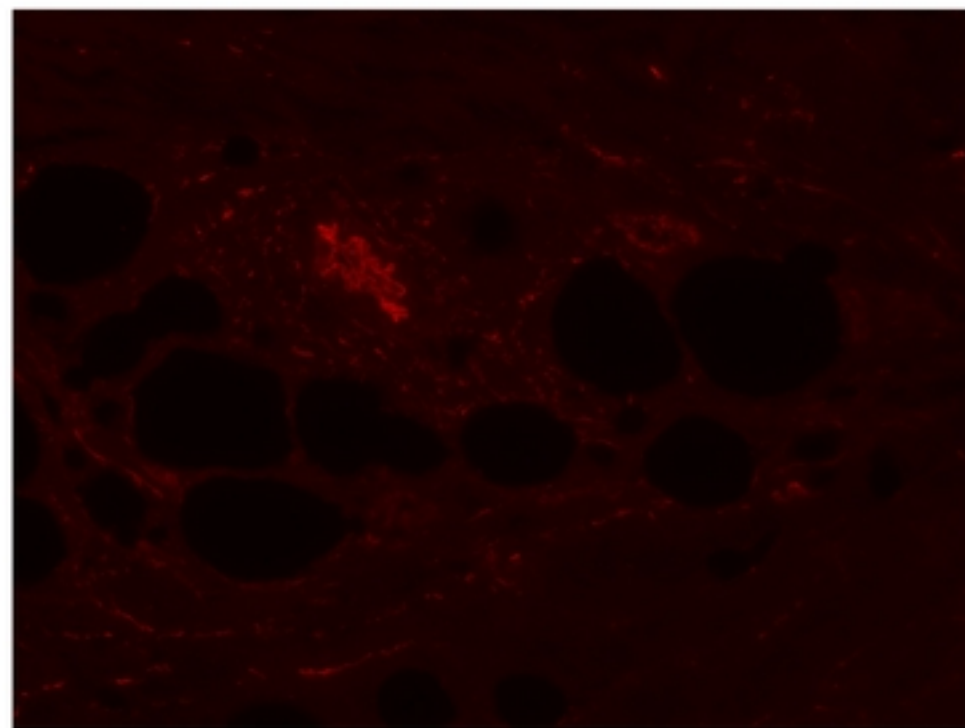
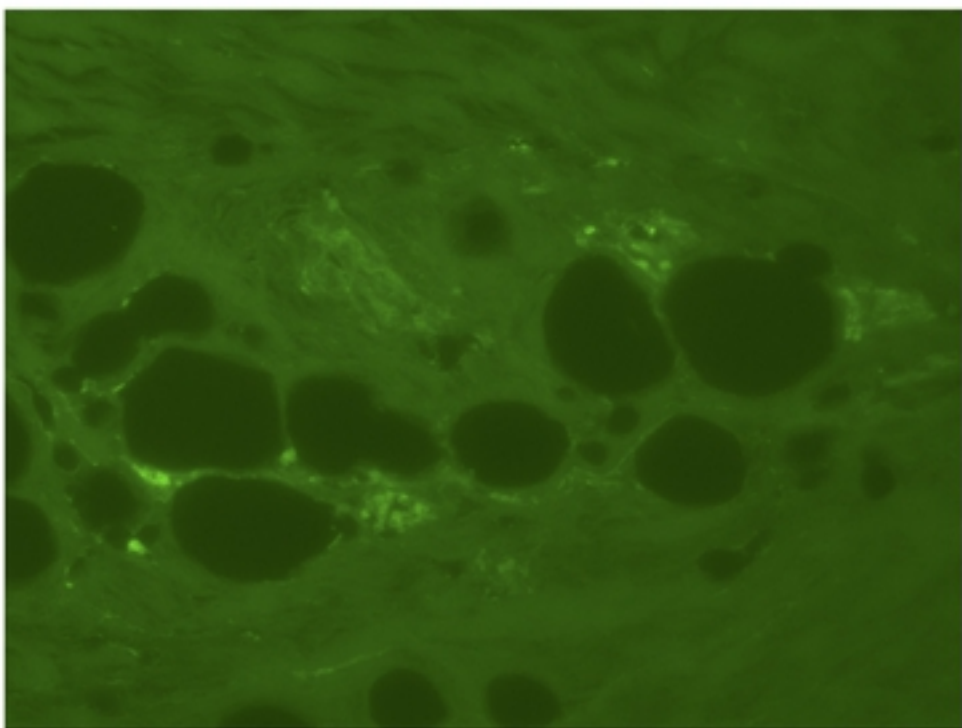


Figure8d

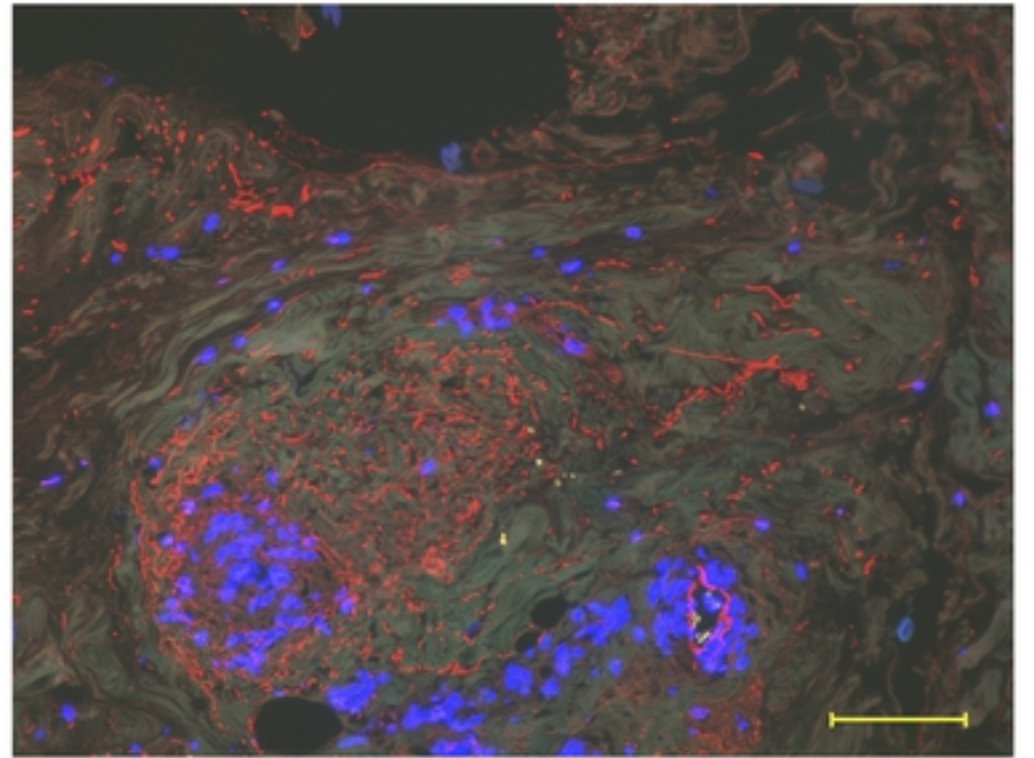
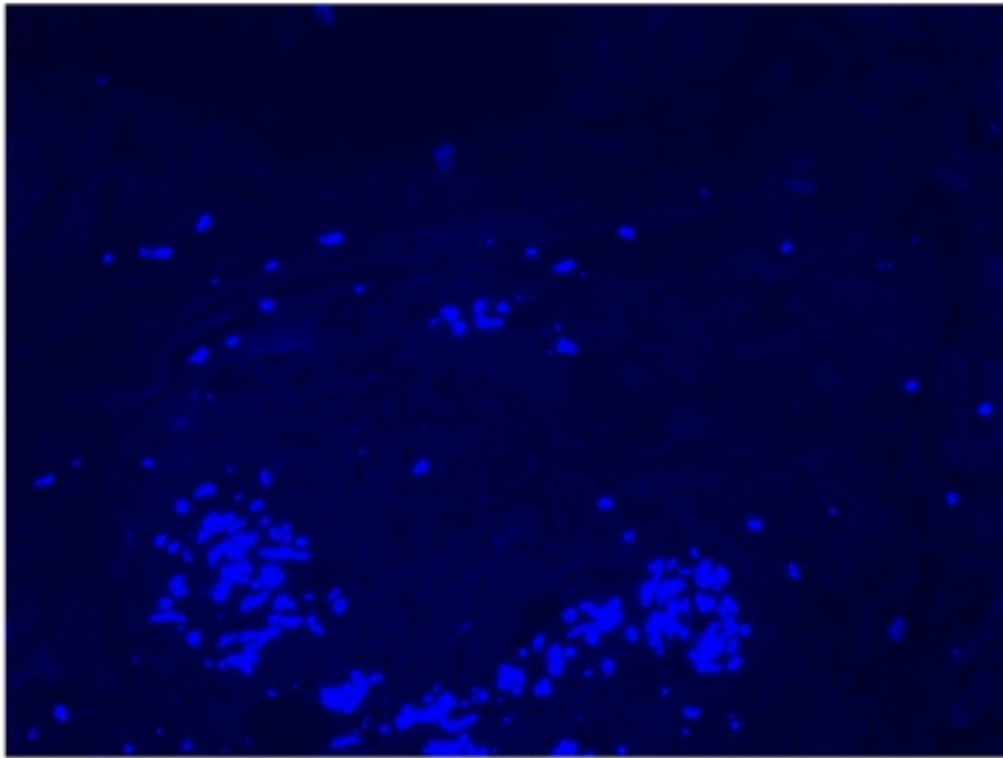
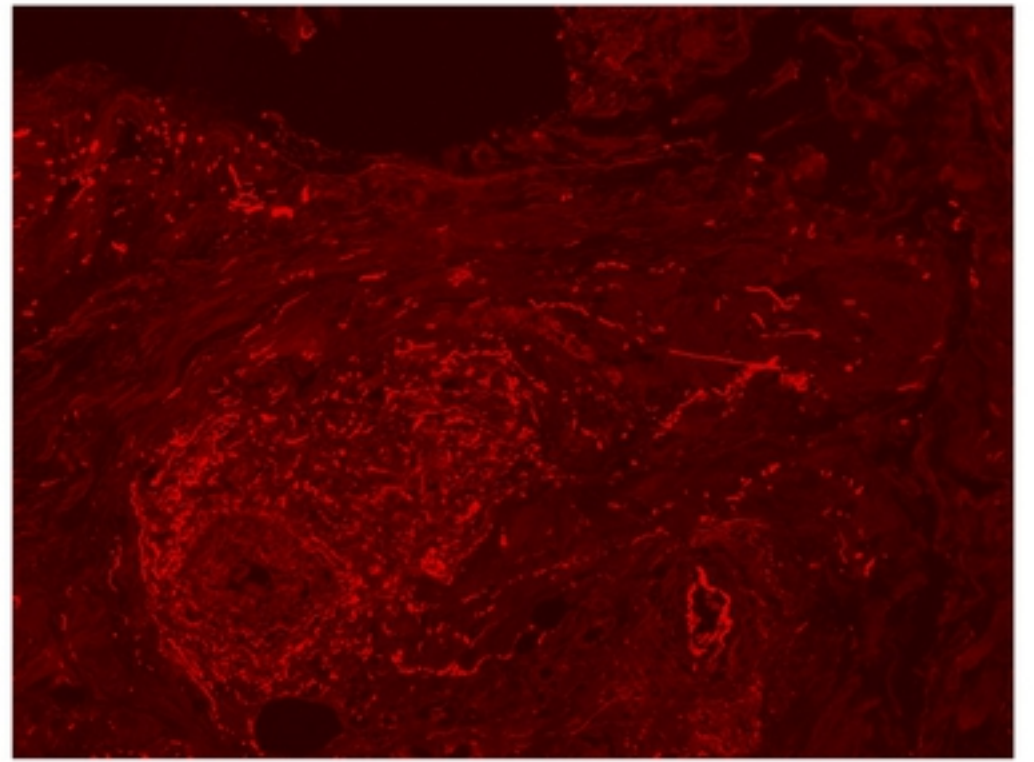
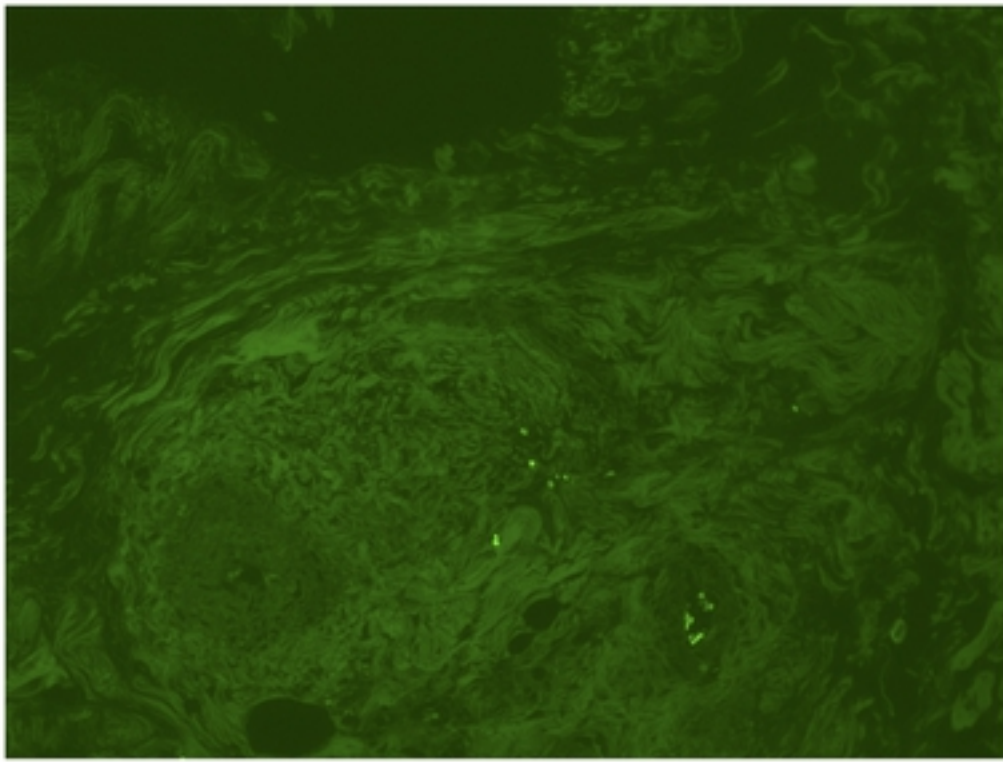


Figure9