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Histological Evaluation of Lymphaticovenular Anastomosis Outcomes in the Rat Experimental Model: Comparison of Cases with Patency and Obstruction

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	Satoshi Onoda.

Histological Evaluation of Lymphaticovenular Anastomosis Outcomes in the Rat Experimental Model: Comparison of Cases with Patency and Obstruction

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Abstract

Background: Lymphaticovenular anastomosis (LVA) plays an important role in the surgical treatment of lymphedema. The outcomes of LVA are evaluated based on changes in edema, and isolated assessment of the anastomosis itself is difficult. We used an animal experimental model to conduct a detailed examination of histological changes associated with LVA and determined the factors important for successful anastomosis.

Methods: The experimental LVA model was created using lumbar lymph ducts and iliolumbar veins of Wistar rats. We performed LVA under a microscope and reviewed postoperative histological changes using optical and electron microscopy. In addition, electron microscopy and histology were used for detailed examination of the area in the vicinity of the anastomotic region in cases with patency and obstruction.

Results: The patency rates immediately after LVA, 1 week after LVA, and 1 month after LVA were 100% (20/20), 70% (14/20), and 65%, respectively. A detailed examination of the anastomotic region with electron microscopy revealed that, in cases with patency, there was no notable transformation of the endothelial cells, which formed a smooth layer. In contrast, in obstruction cases, the corresponding region of the endothelium was irregular in structure.

Conclusion: Vessel obstruction after LVA may be associated with irregular arrangement of the endothelial layer, leading to the exposure of subendothelial tissues and platelet formation. One part of the postoperative changes after LVA and a cause of obstruction were elucidated in this study. Our results may enable improvements in LVA by translating back to real clinical operations.

Introduction

Various treatments for lymphedema can be roughly divided into conservative and surgical treatments. Volume reduction surgery of the diseased limb, such as with liposuction, has been performed for a long time. In recent years, supermicrosurgery[1,2] for vessels ≤1 mm in diameter and lymphatic anastomosis are becoming increasingly common.[3-5] Along with the popularization of supermicrosurgery, lymphaticovenular anastomosis (LVA)[6,7] and vascularized lymphatic node transfer[8-10] that transplant healthy lymph nodes with recipient vessels to the diseased limb have been gradually introduced worldwide. Among these approaches, LVA is the first-choice surgical procedure for the treatment of lymphedema in many institutions because it is relatively safe, fast, and does not require lymph node harvesting, thereby reducing invasiveness.[11-13]

LVA allows the release of lymphatic fluid accumulated in lymph ducts through venous lymphedema. The outcomes of LVA are evaluated based on the changes in edema. Since many factors can contribute to edema, isolated assessment of the anastomosis itself is difficult. In addition, few studies have investigated postoperative LVA courses. Accordingly, many long-term effects of LVA (for example, possible consequences of anastomosis of lymph ducts and veins) remain unknown. In real clinical setting, cases wherein a second surgical intervention allows postoperative histological examination of the LVA site are difficult. Therefore, we used an animal experimental model to conduct a detailed examination of histological changes associated with LVA. Based on a comparison of postoperative courses in cases with long-term anastomotic patency and those with anastomosis obstruction, we determined the factors important for successful anastomosis and identified potential pitfalls.

Materials and Methods

All animal care and studies abided by international laws and policies and were performed in accordance with the Guide for the Care and Use of Laboratory Animals. The experimental model of LVA was created using lumbar lymph ducts and iliolumbar veins from 20 male Wistar rats (Charles River Japan, Yokohama, Japan) weighing 450–500 g (Figure 1). All procedures were performed by a single operator with experience in >100 clinical cases for both microvascular anastomosis for free-flap transfer and LVA.

General anesthesia was induced via intraperitoneal infusion of sodium pentobarbital at a dose of 50–70 mg/kg. Open abdominal surgery was used to identify the veins and lymph ducts, which were carefully isolated and cut. Next, we performed LVA under a microscope to allow the flow of lymphatic fluid from the distal side of the lymph duct into the proximal side of the vein, in a manner similar to normal LVA. End-to-end anastomosis was carried out with six or eight 11-0 nylon stitches (Figure 2). The anastomosed vessels were then placed to avoid pressure from surrounding tissues. As a control, we performed end-to-end anastomosis of lumbar lymph ducts and iliolumbar veins in 10 cases in the same manner.

We reviewed the details of postoperative histological changes using optical and electron microscopy. The anastomotic region, including the lymph duct and the vein, was excised, as shown in Figures 3 and 4. Patent blue staining was utilized for histological examination of the region within ~1 cm from the site of anastomosis. In addition, electron microscopy and histology were used for detailed examination of the area in the vicinity of the anastomotic region in cases with patency and obstruction.

Results

The diameters of the lymph ducts and the veins were within 0.5–0.7 mm and 0.7–0.9 mm, respectively. There was influx of erythrocytes into the lymph ducts in 1 of the 20 cases. This case showed obstruction at the anastomosis site 1 week later. The patency rates immediately after LVA and 1 week after surgery were 100% (20/20) and 70% (14/20), respectively (Table 1). The latter time point was used for all evaluations of obstructed anastomosis (obstruction model, Figure 3). Patency was retained in 13 cases at 1 month postoperatively, and these cases were utilized as the patency model at this time point (patency model, Figure 4). Regarding the 10 controls with end-to-end anastomosis of lumbar lymph ducts, the patency rates immediately after anastomosis, 1 week after surgery, and 1 month after surgery were all 100% (10/10). The patency rate for the 10 controls with end-to-end iliolumbar vein anastomosis was also 100%.

Control cases

Both the vein's anastomotic proximal and distal parts maintained normal configuration, although the anastomotic region was somewhat thicker than was the peripheral zone. There was little transformation of the endothelial cells, which formed a smooth layer. Similar findings were obtained for the lymph ducts of the control cases.

LVA patency cases

Lymph duct and vein

We examined the histological changes using toluidine blue staining and optical microscopy. The vein wall consisted of three levels. An internal elastic membrane was observed to cover several layers of endothelial cells. The tunica media was thin, and the tunica adventitia was present external to a smooth muscle layer. In addition, blood

capillaries, which supply nutrients to the tissues, were identified. Thus, the veins had near-normal anatomy. There was one layer of lymphatic endothelial cells. Several layers of smooth muscles covered the endothelium, but they were much thinner when compared to the corresponding layer in the blood vessels. In addition, lymph valves were present. The structure of the lymph ducts was near-normal (Figure 5).

LVA area

Thin layer cuts revealed the surgical suture (11-0 nylon) in the anastomotic region. On thin layer slices, the internal space of the lymph ducts and veins was preserved, but hyperplasia of the walls was observed in the vicinity of the anastomotic region (Figure 6, yellow oval A). In addition, an aneurysm of the wall was identified on one side (Figure 6, yellow oval B). Detailed examination of the anastomotic region with electron microscopy revealed an increased number of elastic fibers and a thick layer of collagen fibers parallel to the lumen. The presence of an undifferentiated blood vessel indicated regenerative activity. On the other hand, there was little transformation of the endothelial cells, which formed a smooth layer (Figure 7). In the aneurysm area, electron microscopy examination revealed a non-uniform laminar structure, and the sub-endothelial tissue was positioned perpendicularly to the endothelial cells. Additionally, the endothelium level was not smooth, as some cells were aligned towards the lumen (Figure 8).

LVA obstruction cases

Lymph duct and vein

We examined the histological changes using toluidine blue staining and optical microscopy. The continuity of the endothelial layer was partially lost. Endothelial cells and the internal elastic membrane shrank, leading to lumen collapse. Vacuoles were

frequently observed in the veins, partially severing the connection with the perivascular tissue. In addition, the lumen of the lymph duct was filled with red blood cells (Figure 9).

LVA area

The lumen of the vessel was substantially constricted at the level of anastomosis, and both the lymph duct and the vein contained large numbers of blood cells (Figure 10). Red blood cells were especially abundant in the lymph duct. Detailed examination of the anastomotic region performed with electron microscopy revealed that the constriction of the lumen was due to a clot primarily consisting of blood platelets attached to the endothelial cells, and the corresponding region of the endothelium was irregular in structure. In the periphery of the platelets, transformed erythrocytes formed a thick layer in the lumen (Figure 11). On the other hand, although large numbers of erythrocytes were observed in the regions with intact endothelial layers, platelets did not form (Figure 12).

Discussion

Koshima et al. have provided a detailed report about lymphatic vessels in lymphedema in human extremities by ultrastructural observations.[14] However, to the best of our knowledge, this is the first detailed report on histological changes occurring after LVA. Given that the lymph duct and the vein, the two vessels anastomosed during LVA, are radically different histologically, possible transformations in the region of anastomosis, characteristics of the lymph flow, and potential degeneration are of fundamental interest. In clinical practice, LVA is substantially different from a regular free-flap transfer, in that the outcome of the former operation cannot be readily measured in the absence of

significant symptoms. Furthermore, opportunities to examine the state of the anastomosed veins and lymph ducts directly during revision surgery are very rare. Some previous studies demonstrated the presence of considerable postoperative lymphatic flow from the skin surface using lymphatic scintigraphy and indocyanine green (ICG) fluorescence lymphography, but detailed examination of the changes was impossible.[15-16]

Postoperative obstruction mechanism

In this study, all control cases obtained long-term patency. Because the same operator performed all procedures, it is likely that the anastomotic region obstruction was not induced by a technical error, but by other factors. Puckett et al. used a dog experimental model of chronic lymphedema to show that the patency rates 1, 2, and 3 weeks after LVA were 100%, 21%, and 0%, respectively.[17] Gloviczki and colleagues reported patency rates of 80% (8/10), 42% (8/19), and 33% (2/6) at 24 h, 6 weeks, and 8 months after surgery in a study that included 34 dogs.[18] In a study by Maegawa et al., the patency rates after side-to-end LVA measured using ICG fluorescent angiography were 75% at 12 months and 36% at 24 months after the surgery.[19] The observed reduction in patency rate was attributed to a decrease in pressure in the lymph ducts that occurred after the edema subsided. The resulting countercurrent of venous blood into the lymph duct in the anastomotic region diminished the initial beneficial effect of the lymph flow, causing obstruction. We could not verify whether an increase in lymph duct pressure, suggested in previous reports, affected the outcomes of LVA here. However, based on the fact that most cases with patency at postoperative week 1 maintained patency in the long term, we believe that the success or failure of the anastomosis is mainly determined by the surgical procedure itself. It is nevertheless likely that pressure in the

lymph duct contributes to obstruction of the anastomosis region, because we observed a massive influx of erythrocytes into the lymph ducts in the case with obstruction. In the present cases, there was only one case to show countercurrent blood flow to the lymphatic duct. It is possible that a venous return current was inhibited by the vascular clip, which intercepted a blood vessel during the anastomosis. The relationship between pressure and patency should be investigated in the future because of wide variations in venous and lymph duct pressure and the small number of animals in the present study. Some human cases show a postoperative change in skin color that occurs as a result of influx of erythrocytes into the lymph ducts. However, we did not observe such a phenomenon here.

Another factor that may lead to obstruction can be proposed based on the results of the current study. We observed that the transition between the lymph duct and venous endothelial layers was not continuous in the obstruction cases, and the resulting exposure of the subendothelial tissues caused the formation of thrombocytes in the lumen. Platelet formation presumably led to gradual narrowing of the lumen, causing attachment by other blood cells, such as erythrocytes, and resulting in anastomotic obstruction. In contrast, the endothelial transition was smooth, with no exposure of the subendothelial tissue layers, in the patency cases. This factor seems to be important for long-term patency. In this regard, although an outward-facing aneurysm likely caused by the increased pressure in the vessel was observed in the vicinity of the anastomotic region, the endothelial and subendothelial layers were arranged normally, presumably because of the large suture pitch used during the anastomosis. The continuity of the endothelial layer could be the reason for the obtained patency.

Surgical tips and precautions for LVA

From this examination, it seems that preventing protrusion of the subcutaneous tissues into the lumen of the anastomosed vessels is the most important factor for maintaining long-term postoperative patency after LVA. In this regard, it has been shown that peripheral venous angle plasty[20] and side-to-end[21,22] or side-to-side anastomosis with continuous suture, the techniques prone to exposing subcutaneous tissues, are associated with a high probability of postoperative obstruction. Although the consecutive anastomosis-like back wall repair technique[23-25] and the drop-down technique[26] used in normal microsurgery are problem-free, low lymphatic flow and small diameter of the lymph duct compared to that of the vein might pose difficulties in LVA.

We noticed that the lymphatic vessel wall is very fragile and thin in comparison to that of a blood vessel. Because of this, it is hard to confirm the lymphatic duct stump perioperatively, and related errors during the surgical procedure can cause anastomotic obstruction. Therefore, we believe that the most important precautions during LVA are to confirm lymphatic and venous stumps and to turn them outside correctly. Furthermore, extensive suture does not seem to be necessary. Finally, proper surgical technique is of paramount importance, since very minor deviations may lead to postoperative obstruction despite the achievement of perioperative patency.

This study examined anastomotic patency after LVA. However, there are differences between rat and human lymphatics in lymphedema, especially degeneration of smooth muscle cells. In human lymphedema, lymphsclerosis due to ischemia without a feeding vessel is a characteristic finding, but in animals, there is no sclerotic change. It has been suggested that this is the most important factor for obstruction after human LVA. Thus, the present model is adequate for examining details after LVA, but it cannot be used to evaluate the relationship between patency rate and edema formation. Future studies are

needed to address the relationships between patency rate, lymphsclerosis, and edema symptoms.

Conclusion

Histological evaluation of the anastomotic region after LVA in the rat experimental model revealed the factors important for successful anastomosis. It is thought that irregular arrangement of the endothelial layer causes vessel obstruction after LVA by exposing the subendothelial tissues and platelet formation. In contrast, the endothelial transition was smooth, with no exposure of the subendothelial tissue layers in the patency cases. Thus, one part of the postoperative changes after LVA and a cause of obstruction were elucidated in this study. Our results may enable improvements in LVA by translating back to real clinical operations.

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Figure legends

Figure 1. An example of the rat lumbar lymph duct (white arrow) and iliolumbar vein (black arrow).

We used a lumbar lymph duct and the iliolumbar vein in the rat lymphaticovenular anastomosis model.

Figure 2.

Findings after lymphaticovenular anastomosis in a rat model.

We confirmed that lymph fluid flow into the venous side of the anastomotic region was penetrated under the influence of transparent lymph fluid.

Figure 3. The lymphaticovenular anastomosis obstruction model.

We harvested the lymph duct and the vein that had been anastomosed. The anastomotic region (yellow circle) is distinguishable based on the 11-0 nylon marks. A thrombus is visible in the vicinity of the anastomotic region. The schematic at the top illustrates the locations of the vein (a), lymph duct (b), and anastomotic region (c) sections used in the analysis.

Figure 4. The lymphaticovenular anastomosis patency model.

We harvested the lymph duct and the vein that had been anastomosed. The anastomotic region (yellow circle) is distinguishable based on the 11-0 nylon marks. Adipose tissue is visible around the circumference of the anastomotic region. The schematic at the top illustrates the locations of the vein (a), lymph duct (b), and anastomotic region (c)

sections used in the analysis.

Figure 5. Cross-sections of the lymph duct and the vein in a patency case (toluidine blue staining).
There are no blood cells in the vessel. The rat lumbar lymphatic duct is substantially different from the iliolumbar vein in terms of the structure of the endothelial layer, tunica media, and adventitial stratum as well as in terms of mural thickness.

Figure 6. The anastomotic region in a patency case (toluidine blue staining).
The lymph duct is on the right, and the vein is on the left. Wall hyperplasia is present in the transition region (yellow oval A). Varicose deformation is also visible in the anastomosis region (yellow circle B).

Figure 7. Electron microscopy images of the anastomotic region in a patency case; (Left) small magnification, (Center) medium magnification, (Right) high magnification.
Despite the hyperplasia of the wall, the shape of the endothelial cells and the karyomorphism are preserved.

Figure 8. Electron microscopy images of a varicose deformation in the anastomotic region in a patency case; (Left) small magnification, (Center) medium magnification, (Right) high magnification.
Various cells are arranged irregularly in the lumen. The endothelial cells have regular shapes, and the karyomorphism is normal.

Figure 9. Cross-sections of the lymph duct and the vein in a obstruction case (toluidine blue staining).
The lymph duct is filled with blood cells. The wall structure of the vein is collapsed, and the lumen is narrowed.

Figure 10. The anastomotic region in an obstruction case (toluidine blue staining).
The lymph duct is on the right, and the vein is on the left. The lumen diameter is reduced in the transition region (black arrow). In addition, blood cells of various types are present in the lymph duct and the venous lumen.

Figure 11. Electron microscopy images of an anastomotic region in an obstruction case; (Left) small magnification, (Center) medium magnification, (Right) high magnification. Blood platelets adhere to the endothelial cells, forming a clot. The sequence of the layers in the vessel wall is abnormal.

Figure 12. Electron microscopy images of an anastomotic region in an obstruction case; (Left) small magnification, (Center) medium magnification, (Right) large magnification. The lumen is filled with transformed erythroid cells without agglomeration of thrombocytes. The endothelial structure is normal.

Table.1

Table.1 Details of rat experimental LVA model

No.	Lumbar lymph duct （mm）	Iliolumbar vein （mm）	Countercurrent blood flow	Anastmosis patency (AA/1W/1M)
1	0.6	0.8		○/×/×
2	0.6	0.8		○/○/○
3	0.5	0.7		○/×/×
4	0.7	0.8		○/○/○
5	0.6	0.7		○/×/×
6	0.6	0.8		○/○/○
7	0.6	0.9		○/○/×
8	0.6	0.8		○/○/○
9	0.6	0.8	○	○/×/×
10	0.5	0.7		○/○/○
11	0.6	0.8		○/○/○
12	0.7	0.9		○/○/○
13	0.7	0.8		○/○/○
14	0.6	0.7		○/○/○
15	0.6	0.8		○/×/×
16	0.5	0.8		○/○/○
17	0,7	0.9		○/○/○
18	0.7	0.9		○/×/×
19	0.6	0.8		○/○/○
20	0.6	0.9		○/○/○
mean	0.61	0.81		100/ 70/ 65 (%)

LVA: lymphaticovenular anastomosis, AA: Just after anastomosis, 1W: 1 week later, 1M: 1 month later

Figure.1
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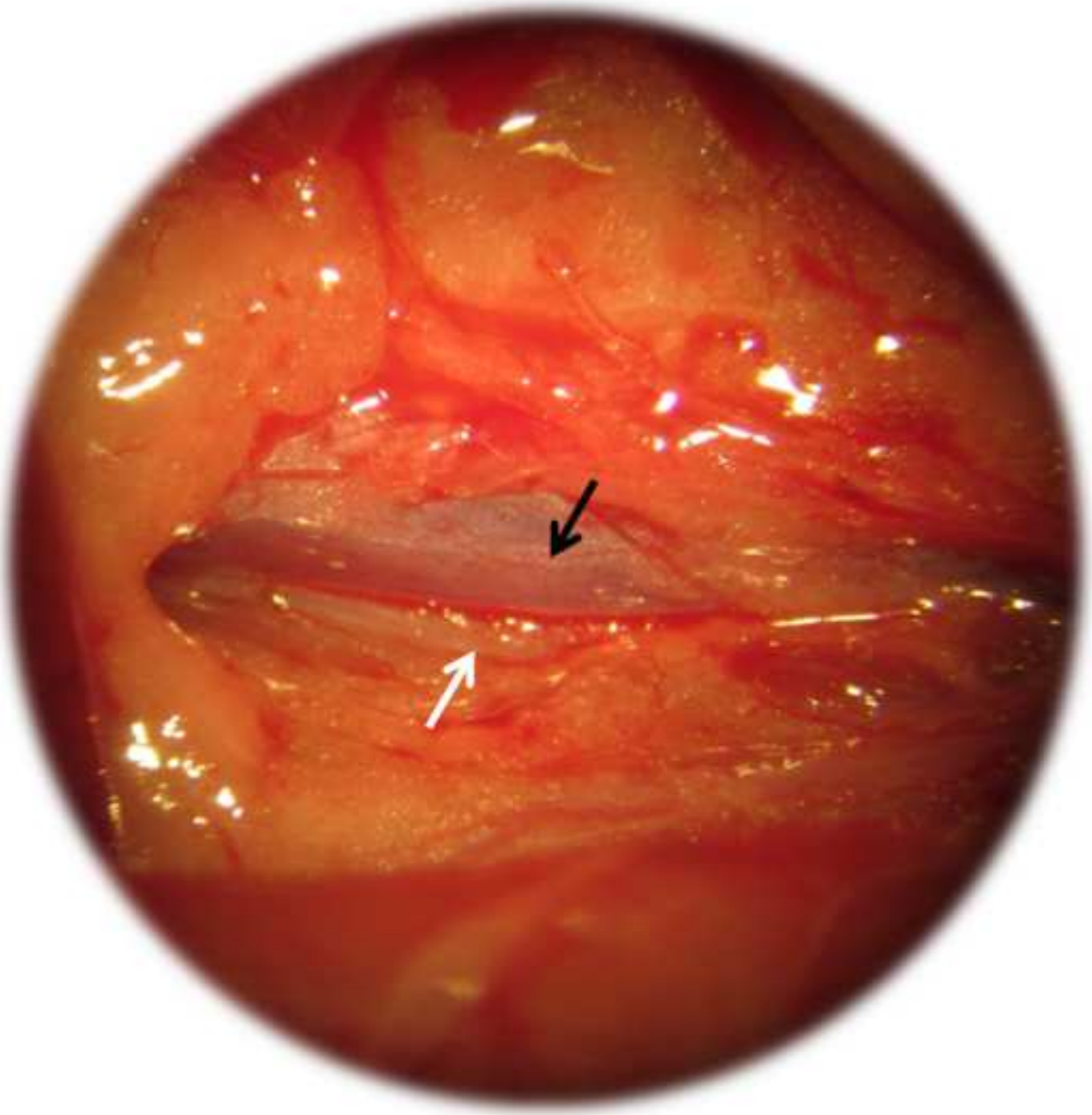

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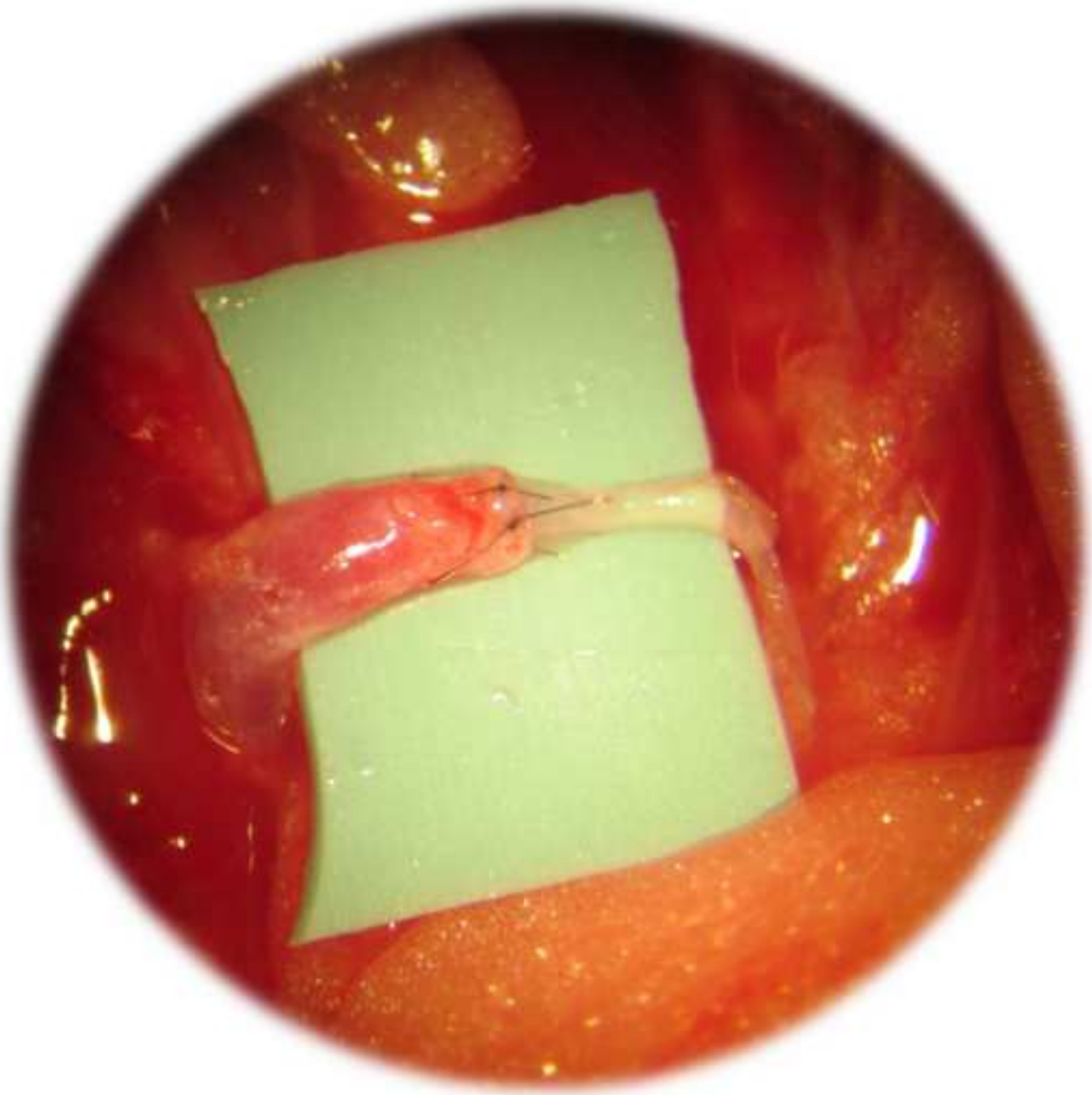

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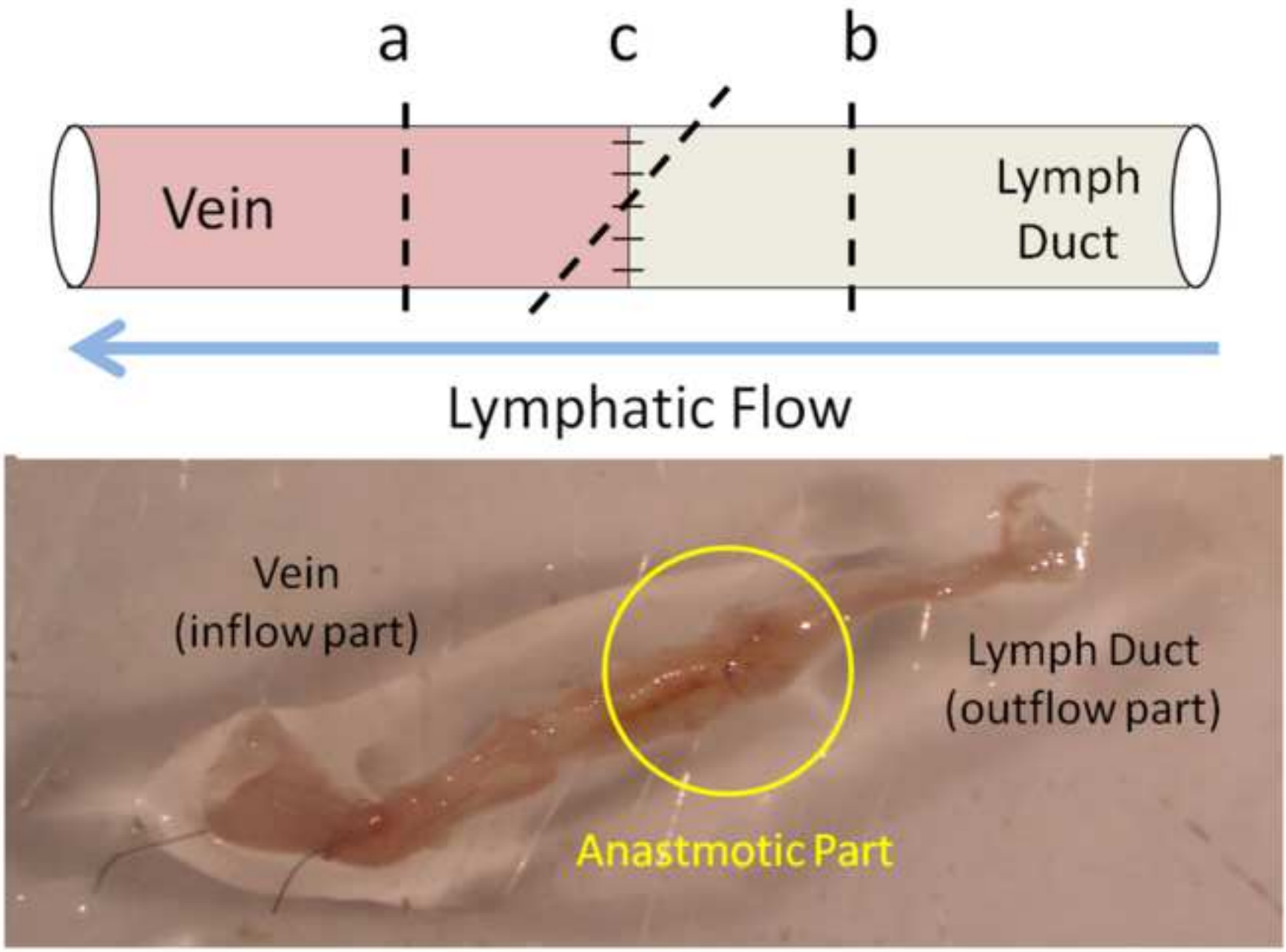

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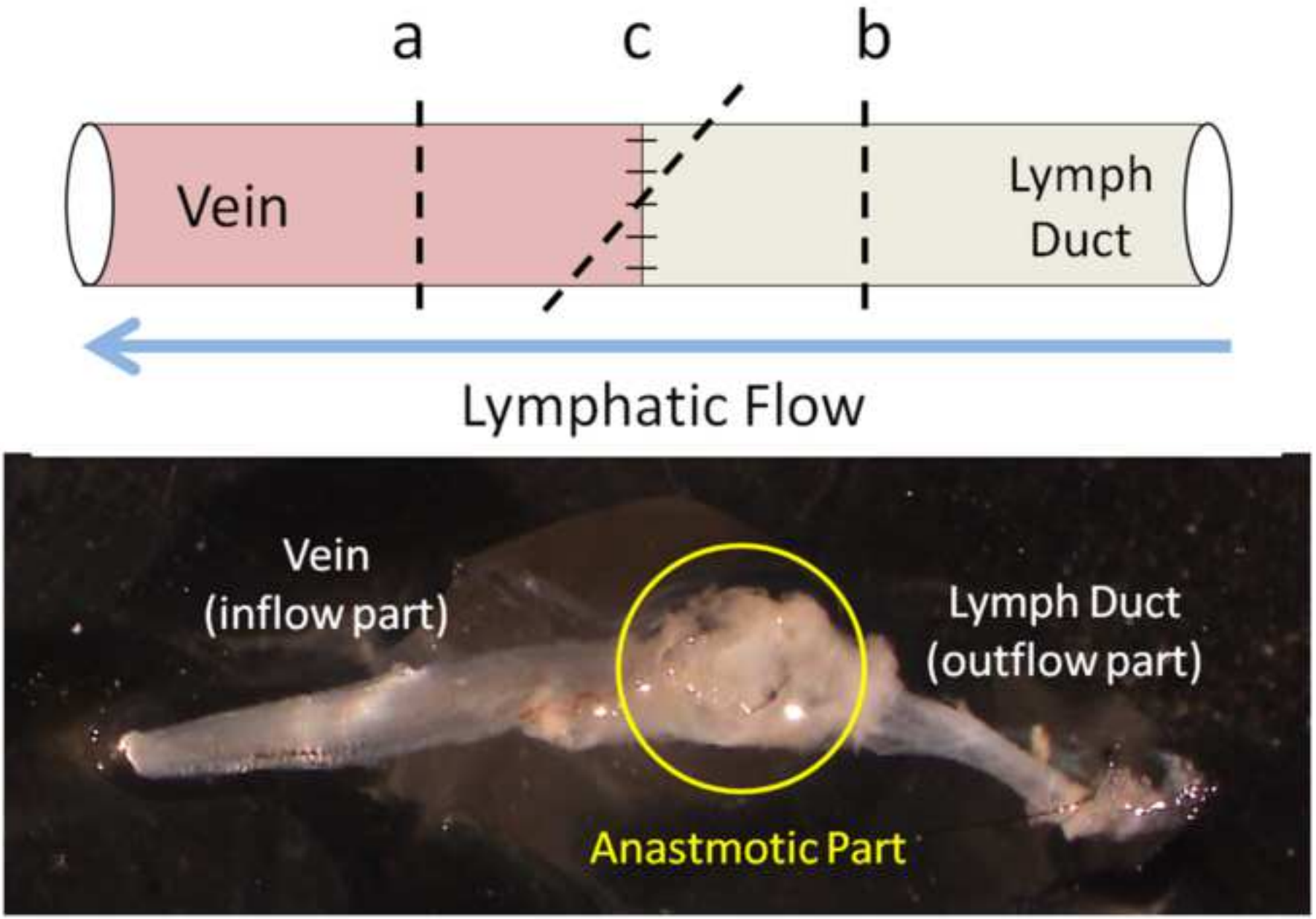

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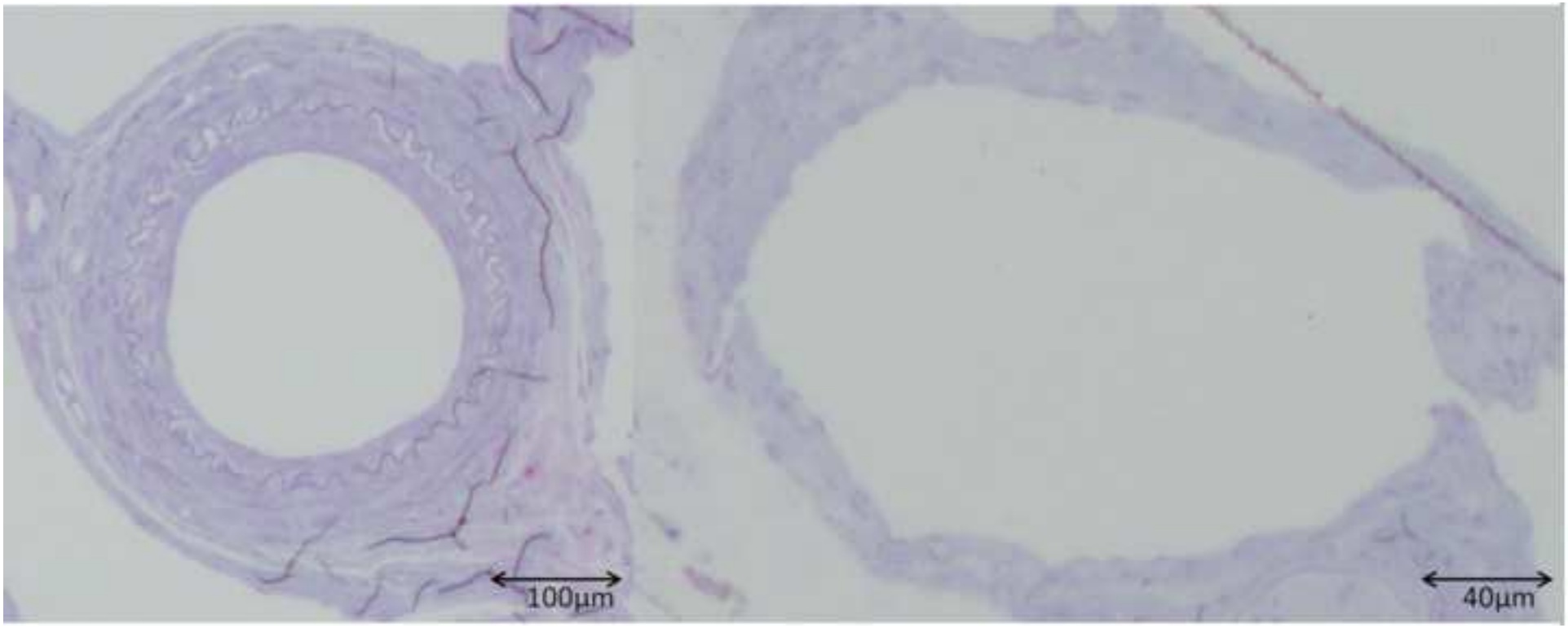

Figure.6
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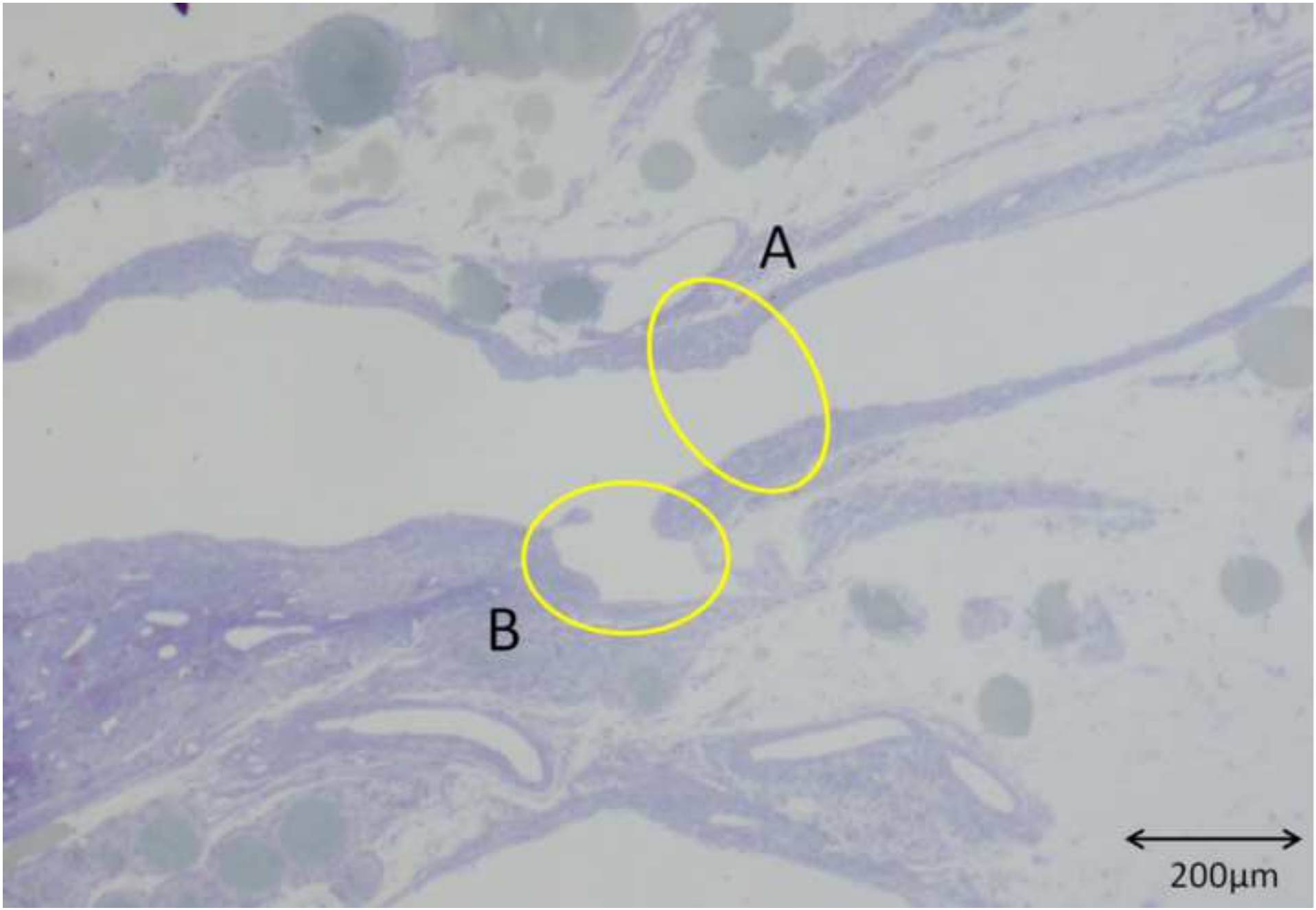

Figure.7
Click here to download Figure: Figure.7.tif

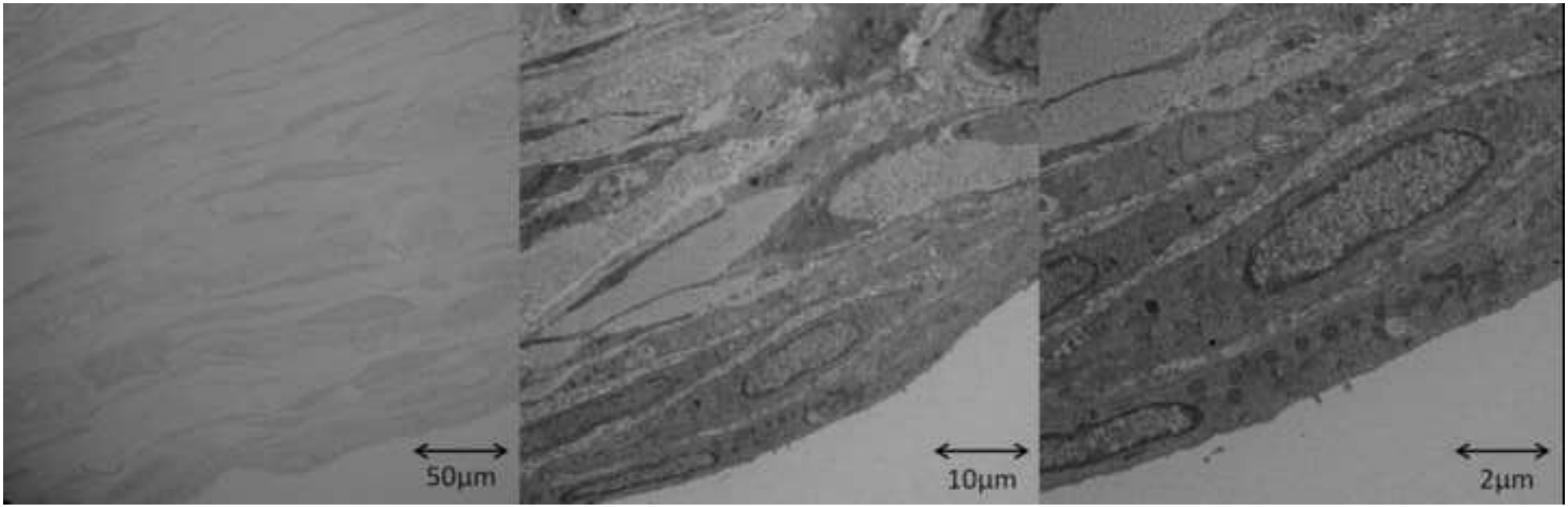

Figure.8
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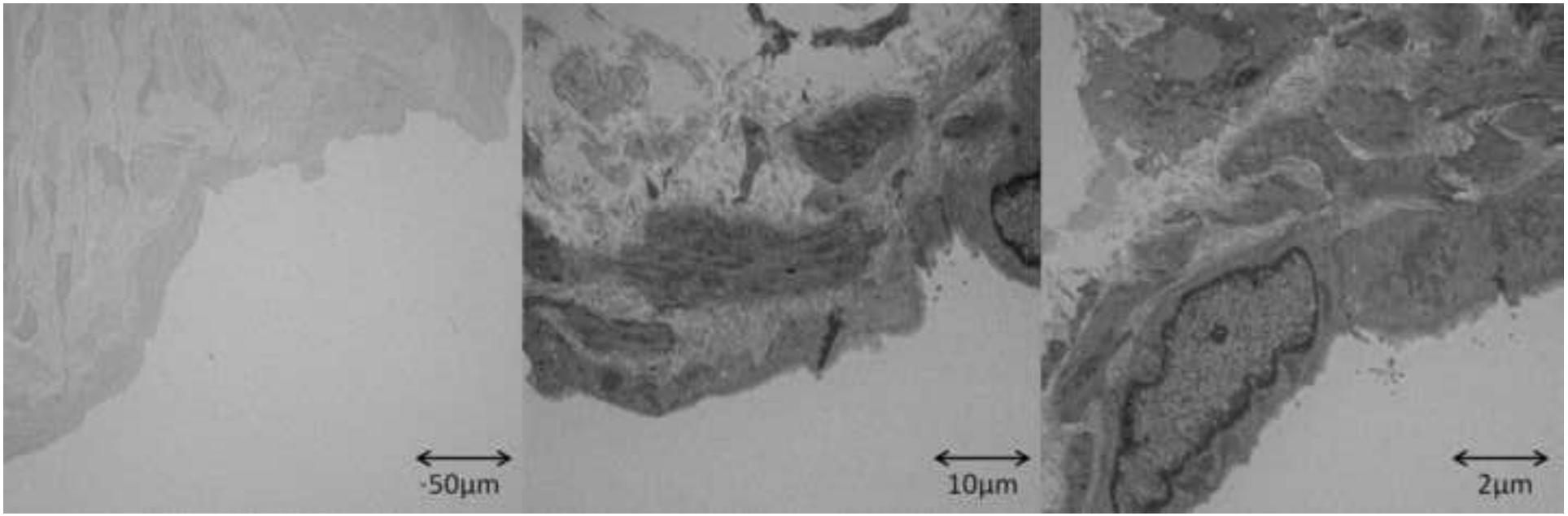

Figure.9
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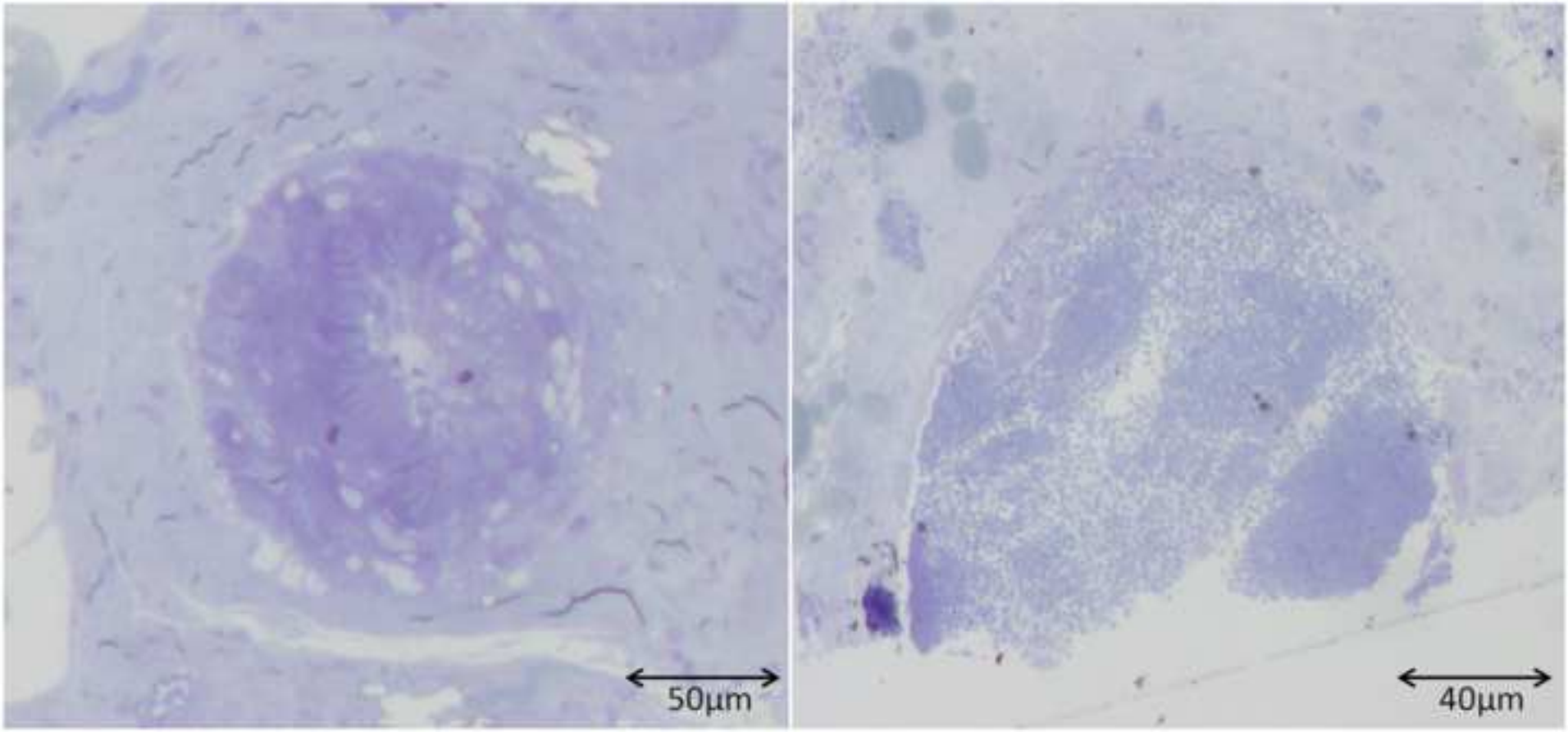

Figure.10
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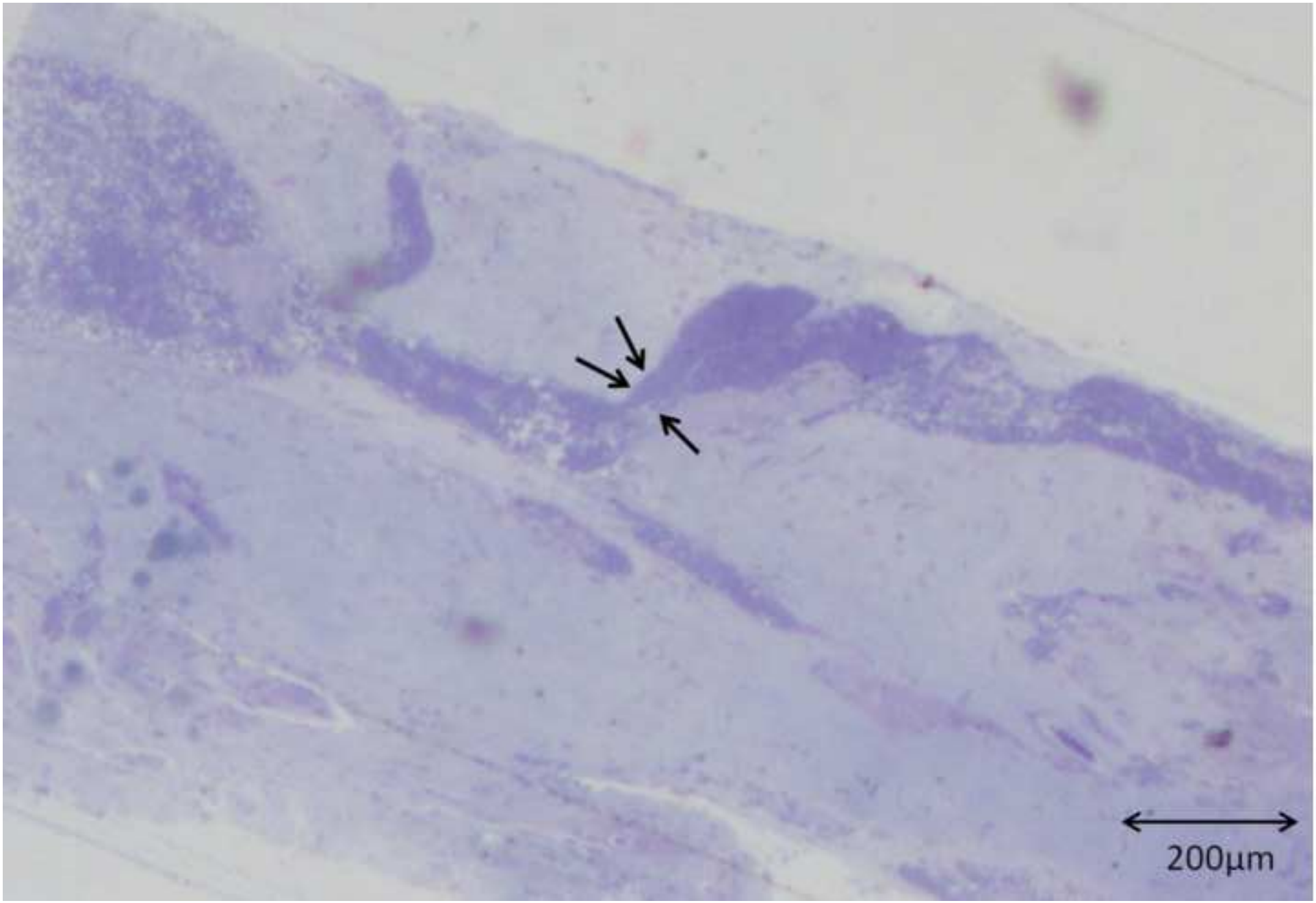

Figure.11
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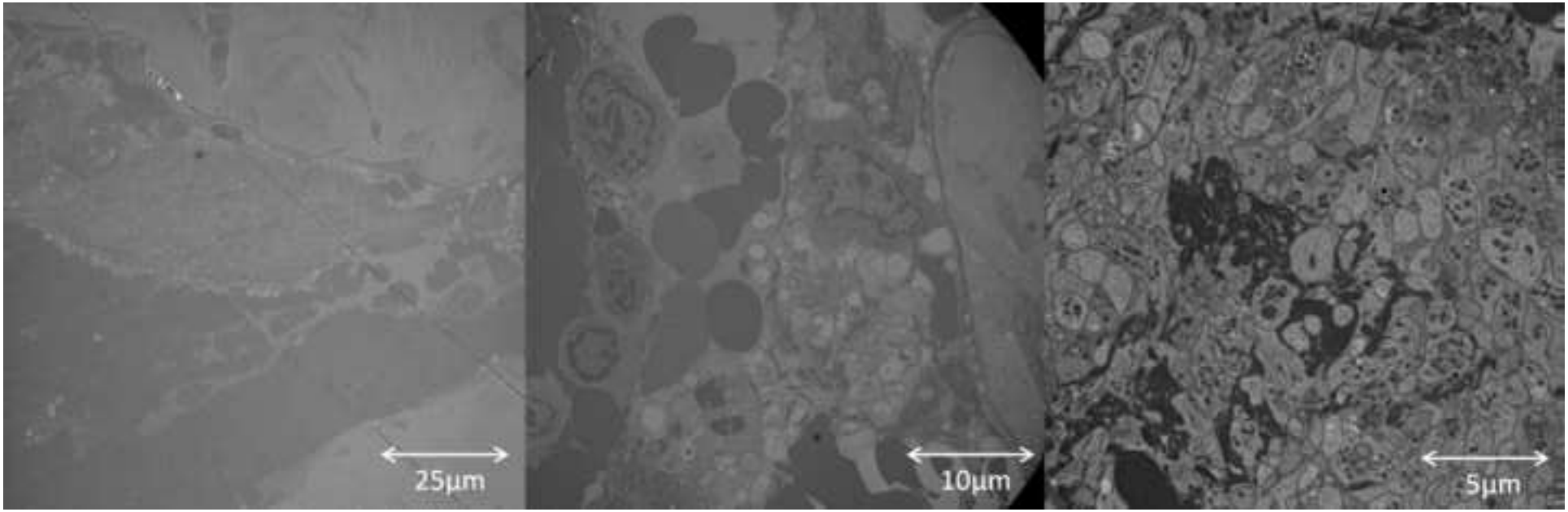

Figure.12
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