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## A simple two-dimensional X-ray angiographic method to examine fine vascular structure using a silicone rubber injection compound

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<b>Author Comments:</b>	<p>16th April, 2018</p> <p>Editorial Board Journal of Visualized Experiments</p> <p>Dear Editor:</p> <p>We wish to re-submit the manuscript titled "A simple two-dimensional X-ray angiographic method to examine fine vascular structure using silicone rubber injection compound." The manuscript ID is JoVE57732R2.</p> <p>We thank you for the thoughtful suggestions and insights. The manuscript has benefited from these insightful suggestions. We look forward to working with you and the reviewers to move this manuscript closer to publication in the Journal of Visualized Experiments.</p> <p>The manuscript has been rechecked and the necessary changes have been made in accordance with editorial comments. The responses to all comments have been prepared and attached herewith. Our changes to our manuscript are in red texts.</p> <p>Thank you for your consideration. I look forward to hearing from you.</p> <p>Sincerely,</p> <p>Seong Oh Park 222-1, Wangsimni-ro, Seongdong-gu, Seoul, Republic of Korea Fax: 82-2-741-3985 Telephone: 82-2-2072-2370 Email: psopark950@gmail.com</p>
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16<sup>th</sup> April, 2018

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*Journal of Visualized Experiments*

Dear Editor:

We wish to re-submit the manuscript titled “A simple two-dimensional X-ray angiographic method to examine fine vascular structure using silicone rubber injection compound.” The manuscript ID is JoVE57732R2.

We thank you for the thoughtful suggestions and insights. The manuscript has benefited from these insightful suggestions. We look forward to working with you and the reviewers to move this manuscript closer to publication in the *Journal of Visualized Experiments*.

The manuscript has been rechecked and the necessary changes have been made in accordance with editorial comments. The responses to all comments have been prepared and attached herewith. Our changes to our manuscript are in red texts.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,

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**TITLE:**

Two-Dimensional X-Ray Angiography to Examine Fine Vascular Structure Using a Silicone Rubber Injection Compound

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**KEYWORDS:**

Angiography, silicone rubber injection compound, lead oxide, barium, flap territory, angiosome, radiopaque material, angiographic agent

**SUMMARY:**

This study presents a simple two-dimensional angiographic method to examine fine vascular structures using a silicone rubber injection compound and soft tissue X-ray system.

**ABSTRACT:**

Angiography is an essential tool for the study of vascular structures in various research fields. The aim of this study is to introduce a simple angiographic method for examining the fine vascular structure of unfixed, fresh tissue using a silicone rubber injection compound and soft tissue X-ray system. This study is especially focused on flap territories used in reconstructive surgery. This study employs angiography with a silicone rubber injection compound in various experimental conditions using Sprague-Dawley rats. First, 15 mL of MV compound and 15 mL of diluent is mixed. Then, 1.5 mL of the curing agent is prepared, and a 24G catheter is cannulated in the common carotid artery of the rat. A three-way stopcock is then connected to a catheter, and the radiopaque agent, after being mixed with the prepared curing agent, is injected immediately without spillage. Finally, as the agent solidifies, the specimen is harvested, and an angiographic image is obtained using a soft tissue X-ray system. This method indicates that high-quality angiography showing fine vascular structures can be easily and simply obtained within a short period of time.

## INTRODUCTION:

Examining vascular structures such as arteries and veins is an important area of interest, particularly in reconstructive surgery. In this field, flap surgery is widely performed. Therefore, angiographic imaging is actively used to study the flap territory, angiosome, and vascular supply of fresh tissue<sup>1</sup>. Specifically, there have been continuous efforts to observe the fine vasculature, including fine vessels such as perforators (vessels emerging from deep vessels reaching the skin), and choke vessels (connecting vessels between adjacent angiosomes)<sup>2</sup>. These two types of vessels are important in the perforator flap reconstruction field and are the main focus of the research<sup>3,4</sup>.

Various materials are used in angiography. First, there is India ink, which is helpful in observing the gross anatomy of blood vessels. However, it is radiolucent, so angiographic images cannot be obtained. The more commonly-used radiopaque materials are lead oxide and barium. However, toxicity is a crucial drawback of lead oxide, and it is inconvenient to use when mixed with water because of its powdered form. Barium is free from toxicity; however, it is not very feasible, as it should be used after dilution. Both of these radiopaque materials cannot cross capillaries; therefore, if a whole vascular structure must be analyzed, it is necessary to inject them into the artery and vein separately<sup>5</sup>. In addition, the two materials cause dye leakage during anatomical dissection, so they should be combined with gelatin. Lead oxide-gelatin and barium-gelatin mixtures take at least one day to solidify<sup>1,6,7</sup>.

Computed tomography (CT) angiography is another widely-used method and can aid in viewing three-dimensional (3D) structures<sup>8</sup>. However, veins cannot be visualized effectively<sup>5</sup>. In this modality, clear visualization of the fine vasculature such as choke veins is difficult, except when using specific equipment. The need for more expensive equipment can be a disadvantage, so CT angiography cannot be utilized in all laboratories. By contrast, the soft tissue X-ray system is relatively cheap and can operate more easily. This system is optimal for viewing soft tissues and can provide higher quality soft tissue images than the simple X-ray system. Although the soft tissue X-ray system itself cannot show 3D images, it can help visualize fine vascular structures more clearly than CT angiography. Therefore, we have used the soft tissue X-ray system in many experiments, particularly in various flap models and basic anatomy<sup>2,9</sup>.

Finally, the use of silicone rubber injection compound angiography has numerous advantages. Because various color agents are prepared, it can be injected and display distinguishable colors such as India ink. Therefore, simultaneously studying the gross anatomy and angiography is possible. It can both pass through capillaries and allow veins to be visualized, making examinations of fine vascular structures possible. Unlike the gelatin mixture, the silicone rubber injection compound solidifies within a short time period, approximately 15 minutes, without any additional procedures. The entire process is summarized in the schematic image in **Figure 1**.

## PROTOCOL:

All procedures, including animal subjects, have been approved by the Institutional Animal Care and Use Committees of Seoul National University Hospital (IACUC No. 10-0184). This protocol is optimized for research on flap vasculature. This example is based on a four-territory flap model

in our previous reports.

## **1. Establishing a Flap Condition**

Note: It is important to generate a vascular change in a rat flap model 4 to 5 days before visible estimation<sup>6,7</sup>.

1.1. Use 7-week-old male Sprague-Dawley rats weighing 200 - 250 g.

1.2. Anesthetize the rats using isoflurane at 3 - 5% for induction and 2 - 2.5% for maintenance. Perform a toe pinch withdrawal reflection test to confirm that the depth of anesthesia is sufficient.

1.3. Shave the trunk using an animal hair clipper and hair removal cream (thioglycolic acid, 80%). Prepare a sterile surgical field with 10% povidone-iodine and a sterile drape to maintain a sterile condition throughout the procedures. Apply a vet ointment to the eyes to prevent dryness.

1.4. Establish the appropriate the flap condition.

1.4.1. Mark a circumferential skin flap design from the lower abdomen to the back, measuring 4 x 12 cm. Locate the center of the flap halfway between the xiphoid process and the penis (**Figure 1**).

1.4.2. Make the incision as marked using a surgical blade.

1.4.3. Dissect the flap using scissors, including the skin and panniculus carnosus.

1.4.4. Dissect around the vascular pedicle [bilateral deep circumflex iliac (DCI) vessels and bilateral superficial inferior epigastric (SIE) vessels] at the lower abdomen and expose the vascular pedicle using a surgical loupe and microsurgical instruments.

1.4.5. Maintain or ligate the vessels depending on the desired conditions.

1.4.6. Divide the flap along the dorsal midline using a surgical blade or scissors.

1.4.7. Lay the flap in its original position and fix it with a skin stapler.

1.4.8. Apply a topical ointment to the surgical wound for 3 days and provide postoperative analgesia by administering meloxicam at a dose of 5 mg/kg orally once per day for 3 days.

1.4.9. Confirm that the rat regains sufficient consciousness to maintain sternal recumbency. Return the rat into the cage and move it to the breeding area.

## **2. Preparation of the Instruments**

2.1. Prepare a 24G catheter and a three-way stopcock.

2.2. Prepare mosquito forceps, small scissors, a surgical scalpel, and a surgical blade.

2.3. Prepare the angiographic agent (silicone rubber injection compound).

2.3.1. Blend the color agent compound with the diluent in the sterile specimen collection cup. Ensure an equal quantity by weight: 15 mL of color agent compound and 15 mL of MV diluent in one rat (Sprague-Dawley rat, 200 - 250 g).

2.3.2. Add the curing agent per 5% weight or volume of the mixture solution immediately before injection: 1.5 mL of curing agent in one rat (Sprague-Dawley rat, 200 - 250 g).

### 3. Rat Artery Preparation

3.1. Use isoflurane to anesthetize the rats (3 - 5% for induction and 2 - 2.5% for maintenance).

3.2. Shave the neck using an animal hair clipper and hair removal cream (thioglycolic acid, 80%). Prepare a sterile surgical field with 10% povidone-iodine and a sterile drape to maintain a sterile condition throughout the procedures. Apply a vet ointment to the eyes to prevent dryness.

3.3. Expose the common carotid artery<sup>10</sup>.

3.3.1. Make a 2-cm midline incision between the scapulae.

3.3.2. Dissect more deeply using mosquito forceps and blunt scissors until the salivary gland complex is exposed.

3.3.3. Retract the salivary gland and bluntly dissect the omohyoid muscle longitudinally.

3.3.4. Dissect around the common carotid artery.

3.4. Hook the cephalic and caudal sides of the common carotid artery with black silk and affix it.

3.4.1. Keep traction to maintain engorgement of the artery.

3.4.2. Prepare one silk tie at the caudal side.

### 4. Cannulation

4.1. Cannulate the prepared carotid artery using a 24G catheter.

4.2. Tighten the pre-made tie in the caudal side and be careful to not remove the catheter during

177 injection.

178  
179 4.3. Prepare the curing agent (step 1.3.2).

180  
181 4.4. Connect the three-way stopcock securely to the inserted catheter.

182  
183 4.4.1. Confirm regurgitated blood into the catheter by adding negative pressure using an empty  
184 syringe.

## 185 186 5. Injection

187  
188 5.1. Inject the silicone rubber injection compound until the color of the eye and foot has changed.

189  
190 Note: The color change should appear as the injected fluid progresses (injection amount is  
191 approximately 25 - 30 mL for each rat).

192  
193 5.2. Lock the three-way stopcock and wait until the agent solidifies.

194  
195 5.2.1. Be careful not to contaminate with the agent, especially when removing the syringe from  
196 the three-way stopcock. Use a protective barrier such as gauze or vinyl to separate the injection  
197 space from its surroundings.

198  
199 CAUTION: Any contamination makes it difficult to analyze the angiographic image because the  
200 compound is radio-opaque.

201  
202 5.2.2. Confirm the cessation of heartbeats and respiration. Stop the anesthesia.

203  
204 5.2.3. Observe the rate of hardness with the remaining agent as a reference (approximately 15  
205 min needed).

## 206 207 6. Harvesting of Specimen

208  
209 6.1. Make an incision using a surgical blade to the panniculus carnosus 1 cm outside the flap to  
210 prevent damage to any vascular structure inside the flap.

211  
212 6.2. Dissect along the previously dissected plane from step 1.4 (under the panniculus carnosus  
213 plane) and harvest the tissue including the flap and vascular pedicle using scissors (the vascular  
214 structure is included in the flap).

215  
216 6.3. Ligate the pedicle of the flap using a 5-0 silk suture and separate the flap from the body. Be  
217 careful not to damage the vascular structure.

## 218 219 7. Capturing the Angiographic Image

220

7.1. Spread out the specimen, ensuring that it does not fold, and gently place it on the surgical drape using forceps.

7.2. Take a radiography image.

7.2.1. Transfer the specimen lying on the film cassette to the sample loading space.

7.2.2. Set the soft tissue X-ray system to 60 kVp, 5 mA, and 5 s exposure.

7.3. Develop the film in a darkroom using an automatic development machine.

7.4. Scan the film at the highest resolution possible.

## 8. Analyzing the Image<sup>6,7,11</sup>

8.1. Distinguish the arteries and veins based on the continuity of flow and diameter.

8.1.1. Start from the inflow of the pedicle artery and focus on the target vessel being examined.

8.1.2. Measure the diameters with software by first opening the image.

8.1.2.1. Click the **Straight** button and draw a line on the scale bar that is the same length.

8.1.2.2. Open the **Analyze | Set scale** menu and enter the value of the scale bar into **Known distance**.

8.1.2.3. Click the **Straight** button and draw a line onto the vessel of which the diameter needs to be measured.

8.1.2.4. Open the **Analyze | Measure** menu and confirm the **Length**.

8.2. Analyze the vascular pattern considering the flap survival area.

## REPRESENTATIVE RESULTS:

Through following this protocol, the flap vascularity of the Sprague-Dawley rat was examined. A circumferential skin flap from the lower abdomen to the back that measured 4 x 12 cm was marked based on our previous reports. Each specimen was in a different vascular condition.

All the flaps were elevated based on the deep circumflex iliac artery (DCIA) and vein and then supercharged with arteries from various locations. Group 1 was the control, group 2 was supercharged with the ipsilateral superficial inferior epigastric artery (SIEA), group 3 was supercharged with the contralateral SIEA, and group 4 was supercharged with the contralateral DCIA. As a result, angiographs of each flap showed different patterns. If the entire flap was divided into four zones as the standard with the main vessel charging the flap territory, the



angiographic agent has reached the main vessel that charges the next distal zone beyond the supercharging artery. The dilated choke vein was also observed, but not seen in normal skin angiography (**Figure 2**).

In other instances, silicone rubber injection angiography clearly showed fine vascular structures. Here, the method is advantageous because the rat vein is so thin and not easily visualized with angiography. High-quality angiographic images displayed dilated choke veins even in specific conditions looking at arterial supply and venous drainage from different sources (e.g., an artery such as the ipsilateral DCIA or vein such as the contralateral superficial inferior epigastric vein) (**Figure 3**).

#### FIGURE LEGENDS:

**Figure 1: Schema of the entire procedure.** This panel shows the preparation of the desired flap condition beforehand and the performance of angiography 4 - 5 days later, using the following process: angiographic agent preparation, injection, specimen harvesting, and image capturing.

**Figure 2: Typical angiography of flaps on postoperative day 4 in the rat skin flap.** The normal skin represents the original four vascular territory flaps in the lower trunk area. The flap includes four vascular territories, including the bilateral deep circumflex iliac (DCI) and superficial inferior epigastric (SIE) vessel territories (yellow square). All groups have a common vascular pedicle, the deep circumflex iliac artery and vein (DCIA&V). Additionally, each flap has a different supercharging artery. Group 2 is supercharged with the ipsilateral superficial inferior epigastric artery (SIEA), group 3 is supercharged with the contralateral SIEA, and group 4 is supercharged with the contralateral DCIA. Group 4 shows different survived flap areas and angiographic patterns. Because the supercharging artery is distal to the original vascular pedicle (DIEA&V), the more distally located major vessel is contrasted by the agent (yellow arrow). Finally, the dilated choke veins that connect the vascular territories are visible (white arrowhead). i = ipsilateral; c = contralateral. Scale bar = 1 cm.

**Figure 3: Other representative results.** The flap shown is supplied by the ipsilateral deep circumflex iliac artery (DCIA) and drained by the contralateral superficial inferior epigastric vein (SIEV). (Upper left) This panel shows the gross photography of the harvested flap. The ipsilateral side shows congestive change, but flap necrosis does not occur. The mid-portion and distal two-thirds of the flap show a complete survival. (Lower left) Angiography shows detailed fine vascular structure including the dilated choke vein, which connects the adjacent vascular territory. (Right) Magnified angiography distinguishes arteries (red arrowheads) and veins (blue arrowheads) by the continuity and diameter of vessels. In general, veins show larger diameters than arteries. DCIA = the deep circumflex iliac artery; SIEV = the superficial inferior epigastric vein; the white arrowhead = the dilated choke vein. Scale bar = 1 cm.

#### DISCUSSION:

Silicone rubber injection compound angiography can be performed easily, does not require expensive equipment, and offers many advantages. In contrast to the preoperative and

intraoperative evaluations of patients, experiments using animals and cadavers can provide details on specific conditions, enabling more diverse and in-depth studies. The flap model using rats is particularly valuable to clinicians because changes in various contexts can be observed before clinical applications<sup>6,7,11</sup>. For example, when supercharging or superdrainage is applied to a long flap, which can resolve distal ischemic necrosis, predicting the outcome in the flap territory is possible; hence, it is possible to increase the survival of flaps by applying these techniques to clinical practice (**Figure 2**). In the case of cadaver dissection, gross anatomic and angiographic evaluations can be performed simultaneously, maximizing the cadaver's use<sup>12</sup>.

The silicone rubber injection compound is easier to handle than other radiopaque materials. Mixing the silicone rubber injection compound and diluent beforehand and adding the curing agent directly before injection are the primary processes involved. As with other imaging methods, care should be taken to avoid spillage during injection. To avoid spillage, we recommend using a secure tie and a three-way stopcock with a catheter after cannulation. If there is spillage, the specimen may become contaminated with the radiopaque agent, lowering its quality as an angiographic image. As discussed, the silicone rubber injection compound is advantageous in that it solidifies quickly after mixing it in, after about 15 min. However, the experiment is typically finished within this time. Therefore, we recommend mixing in only the amount needed for one rat at a time, even when performing multiple experiments. Performance of X-ray imaging after compound solidification should be done before the denaturation of the fresh tissue.

The molecular size of the silicone rubber injection compound is small enough to pass through the capillaries; therefore, there is no need to inject the agent into the vein separately, which is another advantage. On the contrary, the lead oxide-gelatin mixture or barium-gelatin mixture must be injected into veins separately for venous visualization. However, they cannot pass through valves in the veins, so it is necessary to perform serial cannulations and injections. The physiologic antegrade flow from artery to vein can be observed when using silicone rubber injection compounds, yet this characteristic is not observed when using other contrast agents.

Both arteries and veins can be contrasted with the silicone rubber injection compound and can be distinguished according to the continuity of flow and diameter (**Figure 3**). In general, veins show a relatively larger diameter.

One of the disadvantages of performing a 3D analysis using the soft tissue X-ray system is that it is more difficult than using CT angiography<sup>8</sup>. Although stereoscopic radiography can be achieved using the soft tissue X-ray system, it does not produce the high-quality 3D images seen in CT angiography<sup>13</sup>. However, it is still superior for visualizing fine vascular structures. This is particularly noticeable when using the soft tissue X-ray system rather than the plain X-ray system (**Figure 3**). At present, real-time CT angiography is used to observe 3D changes with time flow<sup>14,15</sup>. 3D real-time images cannot be achieved using only the soft tissue X-ray system, but real-time angiography can be achieved through serial imaging using the mobile C-arm system during the injection. Although its image quality is weaker than that of the soft tissue X-ray system for visualizing fine vascular structures, it can enable evaluation of dynamic changes in real time. We

actively recommend this method to researchers who cannot utilize micro-CT or wish to conduct basic research on fine vascular structures such as small veins that are not effectively visualized by micro-CT.

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#### **DISCLOSURES:**

The authors have nothing to disclose.

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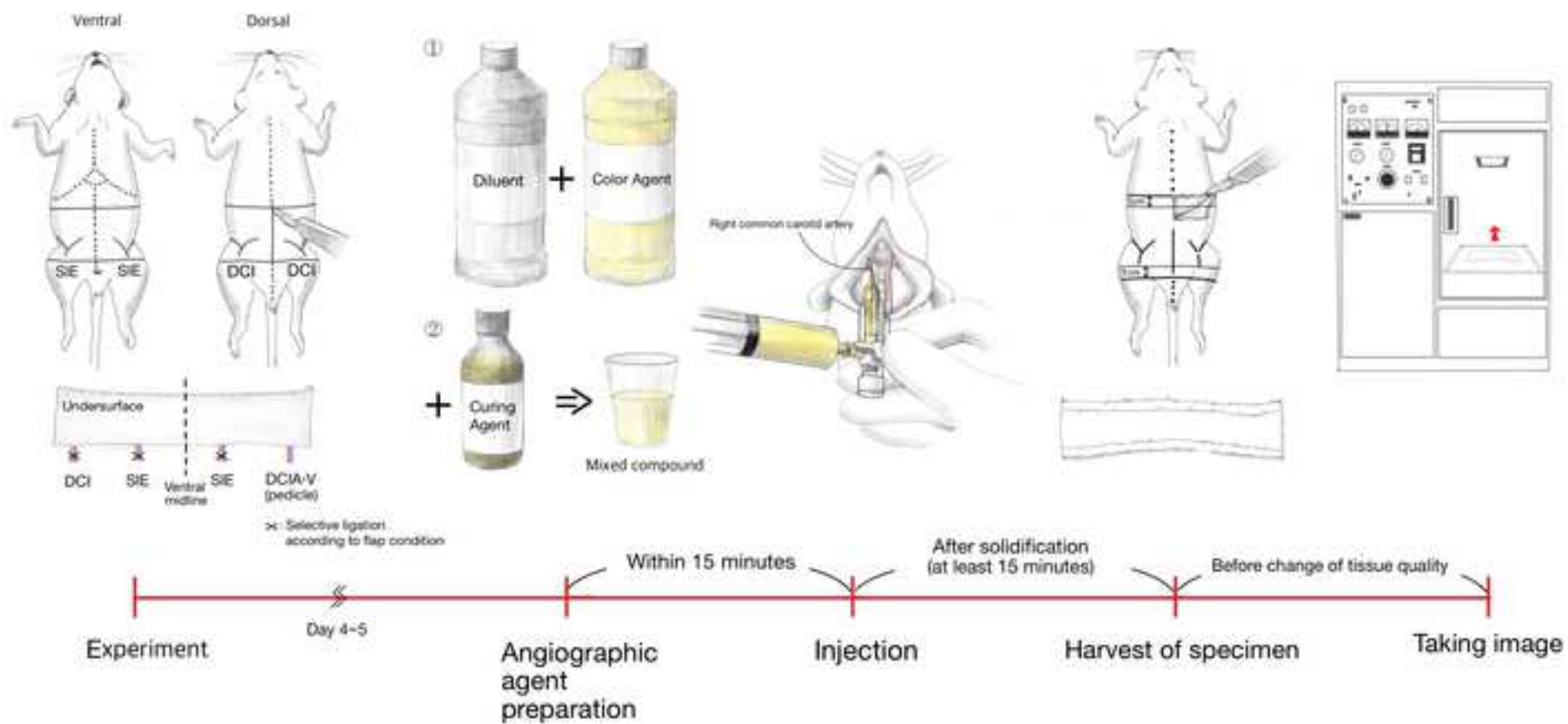


Figure 2

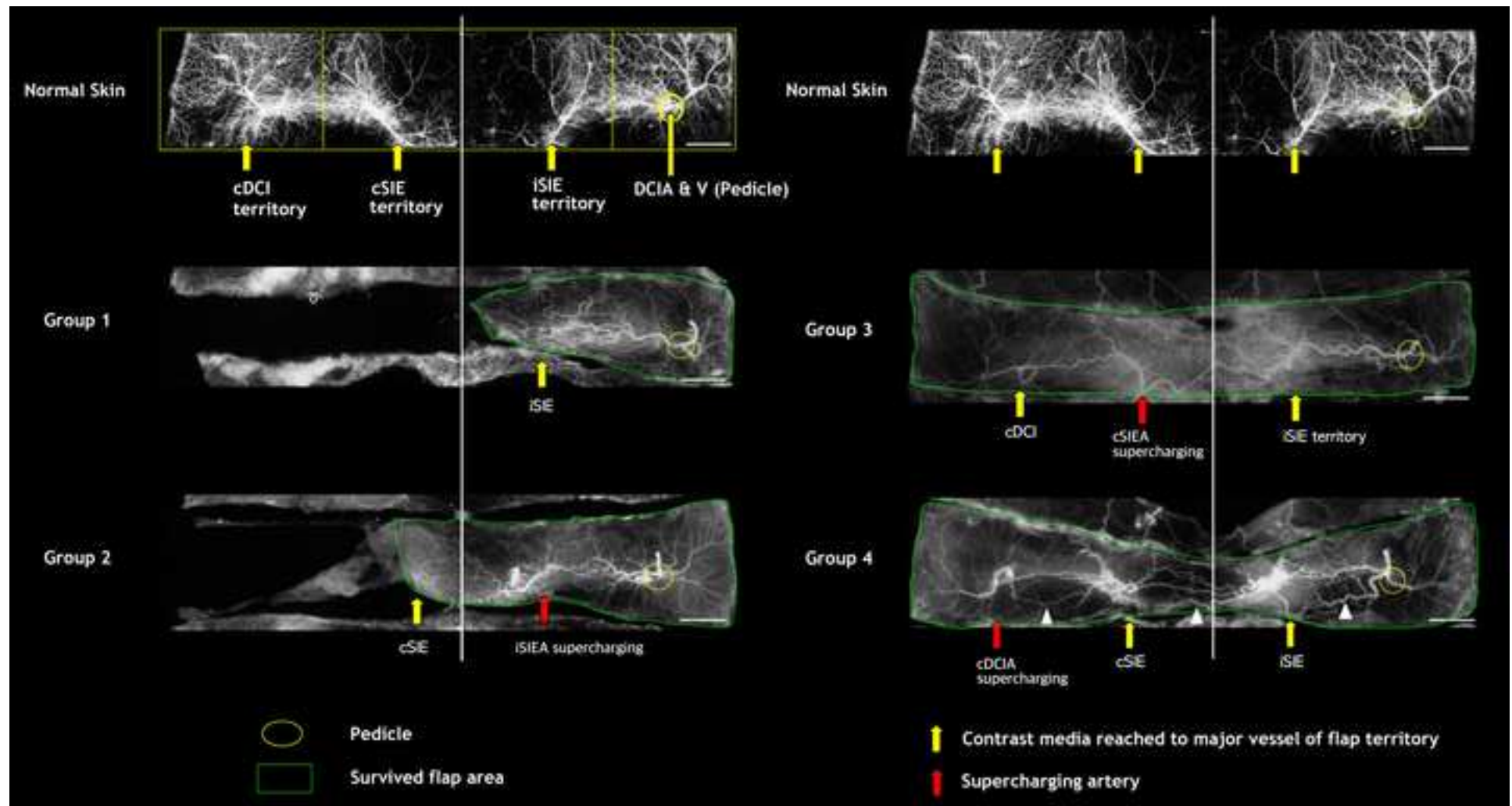
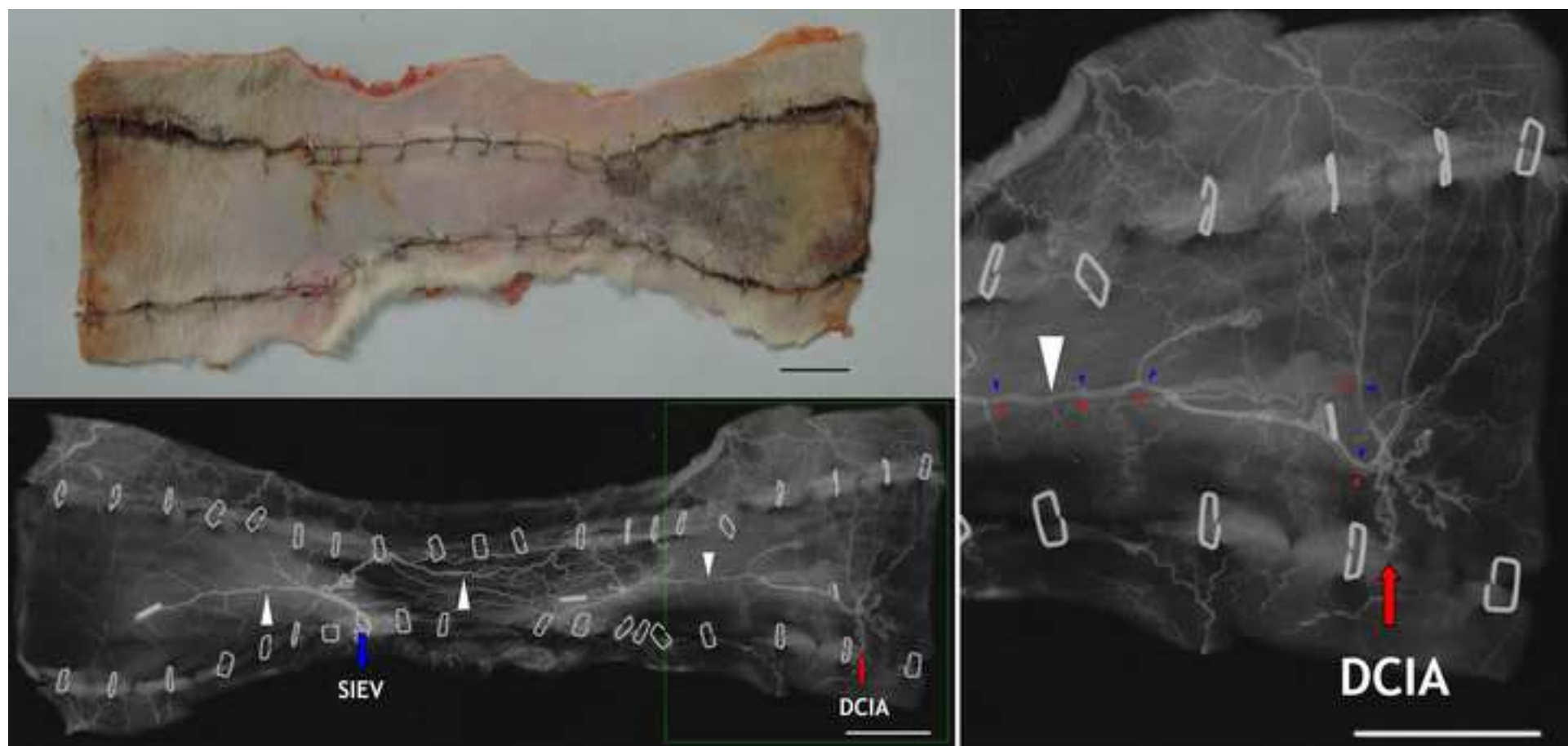


Figure 3



**Name of Material/ Equipment**

MICROFIL Silicone Rubber Injection Compounds
MICROFIL Silicone Rubber Injection Compounds
MICROFIL Silicone Rubber Injection Compounds
MICROFIL Silicone Rubber Injection Compounds
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MICROFIL Silicone Rubber Injection Compounds
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MICROFIL CP-101 For Cast Corrosion Preparations
SOFTEX X-ray film photographing inspection equipment
Film
Automatic Development Machine
Rat
Three-way stopcock
24-guage catheter
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SOFTEX

Fujifilm

Fujifilm

National Institutes of Health

Catalog Number	Comments/Description
MV-112	White color agent
MV-117	Orange color agent
MV-120	Blue color agent
MV-122	Yellow color agent
MV-130	Red color agent
MV-132	Clear agent
MV-Diluent	Diluent
CP-101	Curing agent
CMB-2	Soft tissue x-ray system
Industrial X-ray Film (FR 12x16.5cm)	
FPM 2800	
	Sprague-Dawley rat weighing 200-250 g
	<a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a>



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3. Step 1.1: How to generate the change? Please explain in detail.  
→ The detailed procedure is described in Protocol 1.4. We changed "step 1.1" to "Note."
4. 1.3: How to shave?  
→ We have added more detailed information in Protocol 1.3.
5. 1.4.2: How to dissect? Using what? Please specify the accurate location. What's the size of the incision? How deep is it?  
→ We have added more detailed information in Protocol 1.4.
6. 2.1: Please write this step in imperative tense.  
→ We have revised the sentence according to your advice.
7. 2.2: Please write this step in imperative tense.  
→ We have revised it according to your advice.

8. 3.2: How to expose the common carotid artery? Please explain in detail the surgery procedure.

→ We have added detailed information about the procedure with reference.

9. 4.1: Please write this step in imperative tense.

→ We have revised the sentences.

10. 6.1: How deep is the incision? What is used to make the incision?

→ We have added detailed information about the procedure.

11. 6.2: How to dissect? How to harvest? Please add more details.

→ We have added detailed information about the procedure.

12. 7.3: How to develop the film? What is the film?

→ The method used for developing the film in this study is similar to that used generally. In the past, films were developed manually; however, automatic developing machines have been introduced and are used.

13. Step 8: For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

→ We have added detailed information about the procedure.

14. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please specify the anesthesia or euthanasia method.

→ We performed isoflurane inhalation anesthesia and have described this in

the protocol (Protocol 1.2 and 3.1).

b) Please mention how animals are anesthetized and how proper anesthetization is confirmed.

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c) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

→ We have mentioned this in Protocol 1.3 and 3.2

d) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

→ We have mentioned this in Protocol 1.4.8.

e) Discuss maintenance of sterile conditions during survival surgery.

→ We have mentioned this information in Protocol 1.3. and 3.2.

f) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

→ We have mentioned this in Protocol 1.4.9.

g) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

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15. After you make all changes, if the protocol steps are over 3 pages, please highlight less than 2.75 pages of protocol steps in yellow for filming. Please do not highlight any steps describing euthanasia or anesthesia.

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16. Figure 1: Please add a short description of the figure in addition to the figure title.

→ We have added a short description in the figure legend.