

Journal of Visualized Experiments

Analysis of spinal cord blood supply combining vascular corrosion casting and fluorescence microsphere technique: an aortic surgical animal model --Manuscript Draft--

Manuscript Number:	JoVE56222R2
Full Title:	Analysis of spinal cord blood supply combining vascular corrosion casting and fluorescence microsphere technique: an aortic surgical animal model
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	vascular corrosion casting; fluorescence microspheres; spinal cord blood flow; spinal cord vasculature; collateral blood flow; watershed infarction; thoracic aorta
Manuscript Classifications:	1.2.835.232.834: Spine; 1.7: Cardiovascular System; 1.8.186.854: Spinal Cord; 3.23.550.513: Ischemia
Corresponding Author:	Babak Saravi Heart Center Freiburg University Freiburg, BW GERMANY
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	Babak.Saravi@hotmail.de
Corresponding Author's Institution:	Heart Center Freiburg University
Corresponding Author's Secondary Institution:	
First Author:	Babak Saravi
First Author Secondary Information:	
Other Authors:	Karin Wittmann
	Sonja Krause
	Luisa Puttfarcken
	Ulrich Göbel
	Matthias Siepe
	Friedhelm Beyersdorf
	Fabian Alexander Kari
Order of Authors Secondary Information:	
Abstract:	Spinal cord ischemia after cardiovascular interventions continues to be a devastating problem in modern surgery. The role of intraspinal vascular networks and anterior radiculomedullary arteries (ARMA) in preventing spinal cord ischemia is poorly understood. We are the first to have combined the fluorescence microsphere technique and vascular corrosion cast to investigate spinal cord blood supply in a large animal model. Landrace pigs (n= 30, 35.1 ± 3.9 kg) underwent a lateral thoracotomy under general anesthesia. Fluorescent microspheres were injected into the left atrium and reference sample was aspirated from the descending aorta. Repeated measurements of spinal cord and renal cortical blood flow from the left and right kidneys with three different microsphere colors in 5 pigs were taken to validate reproducibility. Spinal cord blood flow to the upper thoracic (T1-T4), mid-thoracic (T5-T8), lower thoracic (T9-T13) and lumbar (L1-L3) levels were determined. After euthanasia, we carried out selective vascular corrosion cast and counted the left and right ARMAs from levels T1-T13. We observed no alterations in blood flow analysis and fluorescence background noise when using vascular corrosion casting in the same tissue. Repeated measurements of

	cortical renal blood flow and spinal cord blood flow were reproducible. Blood flow analysis of the left and right kidneys revealed a strong correlation ($r=0.94$, $p<0.001$). We detected more left than right ARMAs, with the highest prevalence at T4 ($p<0.05$). The mean number of ARMAs was 8 ± 2 . Their number in the upper thoracic region ranged from 2 to 7 with a mean of 5 ± 1 , while in the lower thoracic region they ranged from 0 to 5 with a mean of 3 ± 1 ($p<0.001$). This study shows that combining fluorescence microsphere technique and vascular corrosion cast is well suited for assessing the blood flow and visualizing the arteries at the same time.
Author Comments:	
Additional Information:	
Question	Response
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

**Analysis of spinal cord blood supply combining vascular corrosion casting and fluorescence
microsphere technique: a feasibility study in an aortic surgical large animal model**

AUTHORS & AFFILIATIONS:

Babak E. Saravi, 1 Karin Wittmann, 1 Sonja Krause, 1 Luisa Puttfarcken, 1 Matthias Siepe, 1
Ulrich Göbel, 2 Friedhelm Beyersdorf, 1 and Fabian A. Kari, 1, *

1 Cardiovascular Surgery, Heart Center Freiburg University, Faculty of Medicine, University of
Freiburg, Germany

2 Anesthesiology and Critical Care, Faculty of Medicine, University of Freiburg, Germany

*Corresponding author. Department of Cardiovascular Surgery, Heart Center Freiburg

University, Hugstetter Strasse 55, 79106 Freiburg, Germany. Tel: +49-761-27024400; fax:

+49-761-27024430; e-mail: fabian.alexander.kari@universitaets-herzzentrum.de (F.A. Kari).

AUTHORS:

Kari, Fabian A.

Cardiovascular Surgery

Heart Center Freiburg University, Faculty of Medicine, University of Freiburg

Freiburg, Germany

Fabian.alexander.kari@universitaets-herzzentrum.de

E. Saravi, Babak

Cardiovascular Surgery

Heart Center Freiburg University, Faculty of Medicine, University of Freiburg

Freiburg, Germany

Babak.saravi@jupiter.uni-freiburg.de

Wittmann, Karin

Cardiovascular Surgery

Heart Center Freiburg University, Faculty of Medicine, University of Freiburg
Freiburg, Germany

Karin.Wittmann@universitaets-herzzentrum.de

Puttfarcken, Luisa

Cardiovascular Surgery

Heart Center Freiburg University, Faculty of Medicine, University of Freiburg
Freiburg, Germany

Luisa.Puttfarcken@universitaets-herzzentrum.de

Krause, Sonja

Cardiovascular Surgery

Heart Center Freiburg University, Faculty of Medicine, University of Freiburg
Freiburg, Germany

Sonja.krause@universitaets-herzzentrum.de

Göbel, Ulrich

Anesthesiology and Intensive Care

University Medical Center Freiburg, Faculty of Medicine, University of Freiburg
Freiburg, Germany

Ulrich.goebel@uniklinik-freiburg.de

Beyersdorf, Friedhelm

Cardiovascular Surgery

Heart Center Freiburg University, Faculty of Medicine, University of Freiburg
Freiburg, Germany

Friedhelm.beyersdorf@universitaets-herzzentrum.de

KEYWORDS:

vascular corrosion casting, fluorescence microspheres, spinal cord blood flow, spinal cord vasculature, collateral blood flow, watershed infarction, thoracic aorta

TITLE:

Analysis of Spinal Cord Blood Supply Combining Vascular Corrosion Casting and Fluorescence Microsphere Technique: A Feasibility Study in an Aortic Surgical Large Animal Model

AUTHORS & AFFILIATIONS:

Babak E. Saravi, Karin Wittmann¹, Sonja Krause¹, Luisa Puttfarcken¹, Matthias Siepe¹, Ulrich Göbel², Friedhelm Beyersdorf¹, Fabian A. Kari¹

¹Cardiovascular Surgery, Heart Center Freiburg University, Faculty of Medicine, University of Freiburg, Freiburg, Germany

²Anesthesiology and Intensive Care, University Medical Center Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany

E-MAIL ADDRESSES:

Babak E. Saravi (Babak.saravi@jupiter.uni-freiburg.de)

Karin Wittmann (Karin.Wittmann@universitaets-herzzentrum.de)

Sonja Krause (Sonja.krause@universitaets-herzzentrum.de)

Luisa Puttfarcken (Luisa.Puttfarcken@universitaets-herzzentrum.de)

Matthias Siepe (Matthias.Siepe@universitaets-herzzentrum.de)

Ulrich Göbel (Ulrich.goebel@uniklinik-freiburg.de)

Friedhelm Beyersdorf (Friedhelm.beyersdorf@universitaets-herzzentrum.de)

Fabian A. Kari (Fabian.alexander.kari@universitaets-herzzentrum.de)

CORRESPONDING AUTHOR:

Fabian A. Kari (Fabian.alexander.kari@universitaets-herzzentrum.de)

Department of Cardiovascular Surgery, Heart Center Freiburg University, Hugstetter Strasse 55, 79106 Freiburg, Germany

Tel: +49-761-27024400; Fax: +49-761-27024430;

KEYWORDS:

Vascular corrosion casting, fluorescence microspheres, spinal cord blood flow, spinal cord vasculature, collateral blood flow, watershed infarction

SHORT ABSTRACT:

This study combines fluorescence microsphere technique and vascular corrosion casting to simultaneously investigate spinal cord blood flow and visualize spinal cord feeding arteries in a large animal model. This model can be employed to investigate morphological vascular alterations and hemodynamic parameters in the same tissue.

LONG ABSTRACT:

Spinal cord ischemia after cardiovascular interventions continues to be a devastating problem in modern surgery. The role of intraspinal vascular networks and anterior radiculomedullary arteries (ARMA) in preventing spinal cord ischemia is poorly understood. We are the first to combine the fluorescence microsphere technique and vascular corrosion cast to investigate

spinal cord blood supply in a large animal model. Landrace pigs ($n = 30$, 35.1 ± 3.9 kg) underwent a lateral thoracotomy. Fluorescent microspheres were injected into the left atrium and a reference sample was aspirated from the descending aorta. Repeated measurements of spinal cord and renal cortical blood flow from the left and right kidneys with three different microsphere colors in 5 pigs were taken to validate reproducibility. Spinal cord blood flow to the upper thoracic (T1–T4), mid-thoracic (T5–T8), lower thoracic (T9–T13), and lumbar (L1–L3) levels were determined. After euthanasia, we carried out selective vascular corrosion cast and counted the left and right ARMAs from levels T1–T13. We observed no alterations in blood flow analysis and fluorescence background noise when using vascular corrosion casting in the same tissue. Repeated measurements of cortical renal blood flow and spinal cord blood flow were reproducible. Blood flow analysis of the left and right kidneys revealed a strong correlation ($r = 0.94$, $p < 0.001$). We detected more left than right ARMAs, with the highest prevalence at T4 ($p < 0.05$). The mean number of ARMAs was 8 ± 2 . Their number in the upper thoracic region ranged from 2 to 7 (mean of 5 ± 1), while in the lower thoracic region they ranged from 0 to 5 (mean of 3 ± 1 ($p < 0.001$)). This study shows that combining fluorescence microsphere technique and vascular corrosion cast is well suited for assessing the blood flow and visualizing the arteries at the same time.

INTRODUCTION:

Microspheres

Since its introduction by Rudolph and Heymann in 1967, microsphere technique has undergone continuous development¹. Today it is the gold standard for measuring regional organ perfusion. Microspheres for blood flow studies are typically 15- μ m-diameter particles labeled with colored, radioactive, or fluorescent substances. When injected into the left atrium, they mix into the central circulation and trigger microembolization in small capillaries (“trapping”). Blood flow is proportional to the number of microspheres in the region of interest. Following introduction of the reference sample method, it became possible to calculate absolute blood flow in mL/min/g by comparing the number of microspheres in the reference sample, aspirated at a predefined rate downstream to the injection site, with the number of microspheres in the region of interest². However, the radioactive microspheres that were first introduced were hazardous for both humans and animals because of the radiation burden. Their expense, especially due to their high disposal costs and large animal experimental models, led to new methods³. Fluorescent microspheres have the advantage of great accuracy, very good spectral separation, high reliability, and low cost compared to radioactive microspheres^{4,5}.

Vascular Corrosion Casting and Spinal Cord Anatomy

Vascular corrosion casting has a long history in describing the morphology of vessels and visualizing small vessels that remain otherwise undetectable by the human eye. With the invention of low viscosity resin in 1970, it became possible to study the microvasculature and distribution of small vessels^{6–8}. Modifications in resin's viscosity helped to obtain highly detailed vascular castings. In combination with scanning electron microscopy, this method can provide a precise image of the endothelial surface of the vessels⁹.

In contrast to these advantages, there are certain sources of error that can affect the casts'

reliability. Although modern polymers have improved the quality of casts, there is still some shrinkage. For example, the average shrinking of the polyurethane-based resin (the same one that we used in our experiments) is reported to be 6.8% after one week¹⁰. Furthermore, extravasation and changes in the surface and surrounding tissue have been mentioned¹¹. However, these observations have not been made with the resin we used¹⁰. Even though combining the fluorescence microsphere technique and vascular corrosion cast is a useful method to describe the anatomy of the vasculature and determine the tissue perfusion in the same model, no simultaneous usage has been reported to our knowledge.

We are the first to combine fluorescence microsphere technique and vascular corrosion casting in an experimental porcine model to determine spinal cord perfusion and visualize ARMA. The ARMAs are branches from segmental intercostal arteries supplying the anterior spinal artery, and vary in number and distribution. 31 somites are formed during embryological development and receive blood from the corresponding segmental arteries through ARMAs, most of which degenerate, and only 4 to 8 of them remain feeding the anterior spinal artery¹². They are therefore crucial for supplying adequate blood flow to the anterior two-thirds of the spinal cord and thus motor functions. Our group's recent investigations suggest that ARMAs play a key role in preventing ischemia after cardiovascular surgery interventions¹³. The Collateral Network Concept introduced by Griep describes intraspinal vascular networks that can prevent acute ischemic conditions if segmental arteries become occluded¹⁴. ARMAs in this case connect the intraspinal collateral system and extraspinal vessels with the anterior spinal artery, thus their number and the maximum distance between them could be an important preoperative risk predictor in aortic surgery^{15,16}. **Figure 1** illustrates the blood supply's schematic to the spinal cord.

PROTOCOL:

This study was conducted at the University Medical Center Freiburg, Freiburg, Germany. Institutional Review Board (IRB) approval was obtained before beginning any experiment. The study animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals and in compliance with the guidelines established by the local German government (Protocol number G 14/39). An experienced veterinarian carried out anesthesia, pain control, perioperative monitoring, and euthanasia.

1. Preparation for Surgery

- 1.1. Allow pigs to house in ventilated rooms and fast them 18 h before surgery. Provide water *ad libitum*.
- 1.2. Premedicate the pigs with an intramuscular injection of ketamine (20 mg/kg) and 0.5 mg/kg of body weight (BW) midazolam.
- 1.3. Insert an 18 G intravenous cannula into an ear and deepen anesthesia with propofol (2–4 mg/kg BW) intravenously (i.v.). Carry out orotracheal intubation with a 6.5 Fr tracheal tube.

Ensure adequate ventilation and oxygenation by ventilation with a positive end-expiratory pressure (PEEP) of 5 cm H₂O, respiratory frequency of 12–14 min⁻¹, and a tidal volume of 8 mL/kg BW.

1.4. Maintain anesthesia with isoflurane 1.5–2% in O₂/Room Air (FiO₂ = 0.6) in combination with fentanyl (5–10 µg/kg/h) and vecuronium (0.2–0.4 mg/kg/h).

1.5. Perform electrocardiogram, pulse oximetry, and temperature monitoring. Use vet ointment on eyes to prevent dryness under anesthesia. Carry out adequate pain control with fentanyl (5–10 µg/kg/h) i.v. and monitor heart rate and pain reactions.

1.6. Under sterile conditions, dissect free the common carotid artery and external jugular vein carefully using scissors, and cannulate with a 3-French-catheter using the Seldinger technique¹⁷. This step is taken to monitor central venous (CVP) and mean arterial pressure (MAP) via pressure transducer and amplifier.

2. Microsphere Injection

2.1. While maintaining sterile conditions, carry out a left posterolateral thoracotomy by an incision in the 5/6 intercostal space using a #10 scalpel blade for the initial incision. Open the situs with scissors and fingers. Open the parietal pleura by an incision and anesthetize the intercostal nerves by injecting 1–2 mL mepivacaine (2%, 20–40 mg). Open the situs by introducing a rib spreader.

2.2. Dissect the thoracic aorta free using scissors, tweezers, and fingers, and introduce a 3-French-catheter into the aorta to withdraw microsphere reference samples. Connect a three-way-stop-cock for blood sampling.

2.3. Open the pericardium using a scissor and insert a 14-G-cannula into the left atrium through the left atrial appendage. Secure the cannula with a 4-0-prolene suture for microsphere injections. Rinse with approximately 10 mL saline to maintain patency for microsphere injection.

2.4. Calculate the minimum number of microspheres to be injected using the formula:

$$N (min) = 400 \times (n) / \left(\frac{Q(organ)}{Q(total)} \right)$$

Where, N (min)= minimum number of microspheres required for the injection, n = total number of organ pieces, Q (organ)/Q (total) = fraction of total cardiac output supplying the organ of interest.

Note: There should be a minimum number of microspheres in the region of interest to ensure highly accurate measurements¹⁸. Here, 2.5 million microspheres were used for the injection.

2.5. Store the vials containing the fluorescent microspheres in a refrigerator at 2–8 °C and protect them from light. Vortex the fluorescence microsphere vials containing 10 mL solution (one million microspheres per mL) for 20 s and place them in a cold ultrasonic water bath for 5 min.

Note: Because the heat generated might damage the microsphere particles, do not leave them in the ultrasonic bath for too long.

2.6. Dilute 2.5 mL (2.5 million) of microspheres with 7.5 mL sodium chloride in a 10-mL plastic syringe.

Note: Carry out injection immediately after aspiration into the syringe. Aggregation of microspheres leads to inaccurate measurements.

2.7. Inject the microsphere solution into the previously introduced left atrial cannula at a steady injection rate lasting 60 s. Inject only in hemodynamically stable pigs to ensure good microsphere distribution in the cardiovascular system and accurate measurements.

2.8. Aspirate the reference blood sample through the aortic catheter with a withdrawal pump at a predefined aspiration rate of 4.55 mL/min into a 20-mL syringe.

2.8.1. Start aspiration 15 s before the microsphere injection, and continue for an additional 180 s for a total of 195 s. This step guarantees that all microspheres are “trapped” in the tissue and reference sample, and can be used to calculate blood flow.

2.8.2. Transfer the blood samples into tubes. Rinse the syringe with 5 mL 2% Tween 80 solution and add it to the tube.

2.9. Repeat steps 2.5–2.8 with fluorescent microspheres of different colors that do not exhibit spectral overlap of excitation and emission wavelengths to obtain blood flow at different time points.

Note: The time points can be chosen according to the experimental setup. Up to 7 different microsphere colors can be used in the same model without performing correction calculations¹⁹. Adequate postsurgical treatment, including treatment of postsurgical pain and recovery conditions are obligatory for survival strategies and long-term experiments. Do not leave an animal unattended until it has regained sufficient consciousness to maintain sternal recumbency. Do not return an animal that has undergone surgery to the company of other animals until it has fully recovered.

2.10. Perform euthanasia using thiopental (20 mg/kg BW i.v.), potassium (5 mmol/kg BW i.v.), and exsanguination by cutting the inferior vena cava under isoflurane anesthesia.

3. Vascular Corrosion Casting

3.1. Immediately after sacrifice, place the animals in supine position.

3.2. Place a 14-French-catheter in the descending aorta, fix it with a 4-0-prolene suture and flush with 500 mL saline with heparin.

3.3. Mix 50 mg blue vascular casting pigment in 50 g casting resin until a dark blue solution is obtained. Mix the dilution solution (74.1 mL ethanol, 10 mL 2-propanol, and distilled water in 100 mL solution) and 5 g hardener into the resin. Adjust the amount of casting material and solvent needed for injection depending on the experimental setup.

Note: Ethylmethylketone or dichloromethane can also be used as a dilution solution. A dilution of up to 40% is recommended.

3.4. Draw the prepared casting material into a 50-mL plastic syringe and immediately inject it manually with high pressure into the aortic catheter.

Note: The use of an injection apparatus enables pressure monitoring. A physiological pressure of 90–120 mmHg is recommended.

3.4.1 During injection, maintain high pressure by closing the catheter manually. Perform adequate suction of casting material through the inferior vena cava vein. After the injection, adjust the operating table for a better distribution into the organ of interest.

4. Autopsy

Note: Autopsy is performed the day after the vascular corrosion casting procedure. Store the pigs in a freezer overnight at approximately -10°C .

4.1. Place the animal in prone position and make a longitudinal incision above the dorsal spine processes. Carryout a midline incision from the cervical region to the sacrum using a #10 scalpel blade.

4.2. Dissect the paraspinal muscles off the vertebral column and expose the spinal cord via laminectomies using a bone Rongeur.

4.3. After removing fatty tissue in the spinal canal using an anatomical tweezer, count the anterior radiculomedullary arteries from segments T1–T13. Identify the segments through the origins of the spinal nerves.

4.4. Dissect the spinal cord at each segment for blood flow analysis using a disposable microtome blade, and put the tissue in 15-mL polypropylene tubes. Perform the dissection in the middle of two consecutive spinal nerves. Do not use polyethylene tubes because the

digesting solution used in the tissue processing will also digest the tubes.

4.5. Expose the left and right kidneys taking a posterior surgical approach between segment T12 and the iliac crest using a scalpel and scissors. Deepen the incision through the latissimus dorsi muscle, and remove fat and parts of the lumbodorsal fascia with a tweezer until reaching the renal fossa.

4.6. After the incision into the renal fascia with a scissor, dissect the kidneys free using a scissor and fingers, and remove. Perform this step after removing the paraspinal muscles (step 4.2). Dissect the outer renal cortical part of the left and right kidneys using a #11 scalpel, put them into the tubes for blood flow analysis, and store them in the dark at room temperature.

Note: Perform this step to validate microsphere distribution and reproducibility of blood flow analysis due to the simultaneous usage of casting material in the same tissue.

5. Tissue Processing

Note: Blood and tissue samples are processed via a modified sedimentation technique for lipid-rich tissues²⁰.

5.1. Allow the samples to rest for 2 weeks in the dark at room temperature (18–22 °C) for autolysis to occur. The samples need not be stored in solution.

5.2. After 2 weeks, place 7 mL of 2.3 M KOH with 0.5% Tween 80 into each tube. Vortex for 20 s, and place in a 50 °C water bath for 48 h.

5.3. After 48 h, centrifuge at 2,000 x g for 20 min at 20 °C; the microspheres are pelleted at this step. Remove the supernatant until there is a volume of 1 mL.

5.4. Next, add 7 mL of Triton X-100 and vortex again. Centrifuge at 2,000 x g for 20 min at 20 °C and discard the supernatant until a volume of approximately 1 mL is obtained.

5.5. To neutralize KOH, add 7 mL of dilute buffer (5.88 g K₂HPO₄ in 200 mL distilled water and 22.9 g K₂HPO₄ in 800 mL distilled water; combine the solutions) and vortex the tubes.

5.6. Following the next centrifugation at 2,000 x g for 20 min at 20 °C, remove all but approximately 150 µL of the supernatant.

Note: This step is crucial. Execute this step very carefully to minimize microsphere loss.

5.7. Finally, add exactly 3 mL of 2-ethoxyethylacetate and allow the tubes to rest for 5 days in the dark at room temperature. This step will release the fluorescent dyes from the microsphere particles.

Note: An exact volume of 2-ethoxyethylacetate is crucial because the fluorescence-intensity measurements depend on the concentration. The tube should be placed in the dark, because the fluorescent dyes are no longer bound to the particles and the fluorescence intensity will weaken due to light exposure (“quenching”).

5.8. After the last centrifugation step at 2,000 x g for 20 min at 20 °C, take out the supernatant and measure the fluorescence intensity in the spectrometer.

Note: A less time-consuming wavelength program can be used to take repeated measurements at different excitation and emission wavelengths according to the microsphere colors used in the experiment. Excitation (Ex) and Emission (Em) wavelength of red, green, and yellow fluorescent microspheres: Red (Ex/Em), green (Ex/Em), yellow (Ex/Em) = 568/595, 455/482, 508/538, respectively.

5.9. Calculate the regional blood flow (mL/min/g) using the following formula:

$$RBF = \frac{F_t}{F_{ref}} \times \frac{R}{g}$$

With RBF: regional blood flow; F_t : fluorescence intensity of tissue sample; F_{ref} : fluorescence intensity of reference sample; R : withdrawal rate of pump; g : tissue weight in grams.

REPRESENTATIVE RESULTS:

Blood Flow Analysis:

We observed no alterations in the blood-flow analysis results and background fluorescence noise when using corrosion-casting material in the same tissue. To prevent background fluorescence with the combined technique, the microsphere colors and inherent background fluorescence of the casting pigment must not exhibit spectral overlap of their excitation and emission wavelengths. Furthermore, spectral overlap with the solvents used for digesting the tissue must be excluded.

Regional blood flow values are shown in **Figure 2**. Blood flow to the mid-thoracic region in the spinal cord was less pronounced than to the upper thoracic ($p < 0.05$), lower thoracic ($p < 0.001$), and lumbar levels ($p < 0.05$). The absolute blood flow values allow a comparison between different timepoints and regions in the tissue of interest. Repeated measurements of spinal cord blood flow values are shown in **Figure 3**. We identified no significant differences in spinal cord perfusion at the three timepoints. The microsphere method thus yielded a reproducible blood flow measurement at different timepoints. Regional cortical renal blood flow in the left and right kidneys is shown in **Figure 4**. A strong correlation was detected between blood flow values from the left and right kidneys ($r = 0.94$, $p < 0.001$). There was no difference in regional renal cortical perfusion among repeated measurements. The high correlation and reproducible blood flow measurements of the kidneys reveal good distribution

of microspheres in the cardiovascular system and their high yield in the tissue-digesting process. A strong difference between the kidneys is evidence of a hemodynamically unstable pig during injection, bad pipetting during tissue digesting, or a faulty microsphere injection or withdrawal of the microsphere reference samples.

Vascular Corrosion Cast:

ARMAs were well perfused with casting material through their entire length (**Figure 5**). A low penetration of the ARMAs is due to the casting material's high viscosity or a low injection pressure. A material of low viscosity will penetrate smaller vessels. The anterior spinal artery, however, exhibited some interruptions in the mid-thoracic region. Such interruption is technical due to the viscosity of the casting material that demonstrates less penetration of the anterior spinal arteries of small diameter in the mid-thoracic region. The ARMAs' number and distribution are shown in **Figure 6**. We counted more left than right ARMAs in all autopsies ($p < 0.05$). Mean number of ARMAs in the pigs was 8 ± 2 . The number of ARMAs in the upper thoracic region ranged from 2 to 7 with a mean of 5 ± 1 , while in the lower thoracic region they ranged from 0 to 5 with a mean of 3 ± 1 ($p < 0.001$). Selective vascular casting allowed visualizing the distribution of these small vessels of the spinal cord. The technique can be combined with scanning electron microscopy to analyze morphological alterations of small vessels.

FIGURE LEGENDS:

Figure 1: Schematic illustrations of the blood supply to the intraspinal and paraspinal vascular system of the spinal cord. Branch points of segment arteries connect the paraspinal with the intraspinal system and consecutive intraspinal systems. ARMAs vary in number and distribution and connect the intraspinal and paraspinal system with the anterior spinal arteries. The paraspinal system is the "sleeping reserve" of blood supply activated by arteriogenic stimuli. It serves as a long-term back-up system, as opposed to the intraspinal collateral system, which is the spinal cord's emergency back-up system as described in reference²¹. Cervicothoracic and lumbosacral inflows to the spinal cord are parts of the Collateral Network Concept¹⁴ (**Illustration 2**). Watershed = Classical Watershed areas of the poor collateralized mid-thoracic spinal cord at T4/T5 and T8/T9²²⁻²⁴. GARMA = great anterior radiculomedullary artery.

Figure 2: Regional spinal cord blood flows in the upper thoracic (T1–T4), mid-thoracic (T5–T8), lower thoracic (T9–T13), and lumbar (L1–L3) level. Spinal cord blood flow is less pronounced in the mid-thoracic region. Results are expressed as mean \pm SD. Student's *t*-tests were performed to determine significant differences. $p < 0.05$ was considered as significant.

Figure 3: Repeated measurements of regional spinal cord blood flow with three different microsphere colors. Results are expressed as mean \pm SD. There is no significant difference in blood flow values between repeated measurements, thus revealing reproducible measurements at different timepoints.

Figure 4: Repeated measurements of regional cortical renal blood flows (mL/min/g) in the

right and left kidneys. Results are expressed as mean \pm SD. A strong correlation ($r = 0.94$, $p < 0.001$) between the left and right kidneys and reproducible blood flow values indicate good distribution of microspheres in the cardiovascular system.

Figure 5: Visualizing ARMAs via vascular corrosion casting. Visualizing the connection between the anterior radiculomedullary arteries and anterior spinal artery via vascular corrosion casting. ARMAs vary in number and distribution and connect intraspinal and extraspinal vessels with the anterior spinal artery. **A** = anterior spinal artery; **B** = anterior radiculomedullary arteries

Figure 6: Distribution of anterior radiculomedullary arteries. This graph illustrates all the anterior radiculomedullary arteries identified between T1–T13 in 30 autopsies via vascular corrosion casting. The highest prevalence of left and right ARMAs was found at level T4.

DISCUSSION:

We are the first to have combined the fluorescence microsphere technique and vascular corrosion cast in this feasibility study to visualize spinal cord vasculature and determine spinal cord blood flow at the same time.

The spinal cord's thoracic region has some important features that must be considered in cardiovascular surgery. The anterior spinal artery's supply of blood through the anterior radiculomedullary arteries leaves watershed areas with decreased blood flow next to the regions in which extraspinal and intraspinal vessels overlap²². The spinal cord's mid-thoracic region in this case has classic watershed areas vulnerable to ischemic damage around segments T4/T5 and T8/T9^{23–25}. Furthermore, the thoracic region reveals the largest space between the ARMAs, and collateral blood flow in this section is reportedly low^{12,26}. The risk for spinal cord ischemia therefore rises when intercostal arteries are occluded, because the collateral system is incapable of providing sufficient blood flow to this area²⁷. Furthermore, the anterior spinal artery is reported to be narrowest in the mid-thoracic region, increasing the risk for infarction in this area.²⁸ The casting material we used penetrated the anterior spinal artery in the mid-thoracic area the least, thus verifying previous findings. The interruptions in our experiments were technical in nature due to the anterior spinal artery's small diameter in the mid-thoracic region and due to the casting material's viscosity. In one study, we carried out a simulated "frozen elephant trunk procedure" (FET) by occluding thoracic segment arteries and interrupting collateral inflow into the epidural arcades to investigate histological changes in the thoracic region¹³. We found out that the 3 h postoperative observation of ischemia was too short to permit the observance of any histological tissue changes. Further long-term experiments could help us better understand the findings previously mentioned.

This study supports the existence of a constant anterior radiculomedullary artery, referred to as the "Artery of von Haller" at level T4, as opposed to the "Artery of Adamkiewicz", which often originates between T8–L3^{29,30}. This fact should be considered in aortic surgery when segmental arteries in this area are occluded because of the watershed zone downstream of this region^{23,31}. Furthermore, the upper thoracic region has more ARMAs than the mid-thoracic and lower thoracic segments. These findings support the theory of a higher ischemia risk in these regions,

as other studies have documented^{23,24,30,32,33}. The regression of ARMAs has been described as being prominent in the caudal region, where the Artery of Adamkiewicz often remains as the largest ARMA^{27,29,34}.

The combined method applied in this study was reproducible in blood flow analyses, although the vascular corrosion cast had been used in the same tissue. The blood flow values we measured in the pig resemble those described before³⁵. The number and distribution of ARMAs in the pig resemble the values documented in humans³⁰. Krucker *et al.* reported some background fluorescence of casting material, but we detected no spectral overlaps with the microsphere colors used in our experiment¹⁰. However, blue microspheres are not recommended, as background fluorescence noise has been reported when using the digesting solution to release the microsphere dyes from the particles and blue pigment for vascular corrosion cast⁵. This combined technique has several limitations that should be addressed. The microsphere method is prone to errors, which can influence the blood flow values calculated. The injection of microspheres in hemodynamically unstable pigs leads to poor distribution of microspheres in the cardiovascular system and therefore inaccurate blood flow values. Furthermore, the reference sample needs to be withdrawn at a steady withdrawal rate for blood flow calculation. Any irregularities in the withdrawal pump lead to inaccurate blood flow calculations. The time-consuming processing of the samples is an important limitation, as each process step can lead to inaccurate blood flow calculations. However, an internal standard can be used with a predefined number of microsphere colors that are not used in the experiments before beginning processing. Low extraction rates of the internal standard can be due to faulty pipetting and an inaccurate digesting-solution volume (step 5.7). However, employing the sedimentation technique enables samples to be processed in one tube, minimizing the loss of microsphere particles²⁰. Finally, the microsphere colors used in the experiment must not exhibit spectral overlap with the vascular casting pigment, the tissue of interest, or the solutions used to process the samples. We recommended making “test samples” with tissue and digesting solution without microsphere particles to measure the tissue’s background fluorescence.

Improvements in perioperative risk management associated with thoracoabdominal aneurysm repair have significantly reduced the incidence of spinal cord ischemia. Cerebrospinal fluid drainage, neuromonitoring using somatosensory-evoked potentials (SSEPs) and motor evoked potentials (MEPs), monitoring of spinal cord perfusion pressure, distal aortic perfusion, and the preoperative visualization of collateral blood flow and ARMAs are important factors for the improving spinal cord risk management. However, preoperative risk management via magnet resonance angiography (MRA) tends to focus on just one ARMA, the great anterior radiculomedullary artery (GARMA). Our group’s recent investigation found that the distance between ARMAs in the thoracic region plays a key role in neurological outcomes after the FET procedure¹³. A large distance between ARMAs led to insufficient reactive hyperemia after acute ischemic settings. Furthermore, we discovered that the number of ARMAs correlates with the decrease in spinal cord vascular resistance and therefore reactive hyperemia after ischemic conditions (unpublished data). Physiologically, we detected no significant correlation between the number of ARMAs and spinal cord blood flow. ARMAs seem to be the collateral means of providing rapid and sufficient reactive hyperemia when the spinal cord is affected by ischemia.

Preoperative visualization of the number and distribution of ARMAs and the collateral pathways could therefore function as an important preoperative risk predictor. In this case assessing the spinal cord blood flow and vasculature is essential for determining the risk. This combined method can be used for preclinical work to investigate tissue blood flow and vasculature. It enables the investigation of absolute blood flow at different time points, but not real-time measurements. Intra-arterial catheter angiography makes highly detailed images to visualize collateral pathways and feeding vessels possible³⁶. However, it is an invasive method associated with the risk for iatrogenic paraplegia itself. Furthermore, it is incapable of visualizing all collateral pathways and ARMAs simultaneously, making more sessions necessary, thus raising the risk for iatrogenic paraplegia^{37,38}. Non-invasive imaging of the GARMA via computer tomography and MRA has improved recently^{39–41}. The assessment of collateral pathways with MRA allowed the assessment of important relations between collateral pathways and spinal cord functions. Backes *et al.* found a significant correlation between the existence of collateral pathways visualized with MRA and a negative predictive value of spinal cord function after cross-clamping in the corresponding aortic area⁴². Further research on collateral pathways and their visualization could help us create individual risk algorithms preoperatively.

We postulate that the combination of fluorescence microsphere technique and vascular corrosion cast can be used in the same tissue to analyze the vascular system and hemodynamics. Furthermore, we call for more research on the role of the anterior radiculomedullary arteries and intraspinal collateral system to lower the rate of spinal cord ischemia after cardiovascular interventions.

ACKNOWLEDGMENTS:

This study was funded by the Deutsche Forschungsgemeinschaft, German Research Foundation (DFG) Grant Nr. KA 3872/2-1. We thank Dr. Amir Alkalak for providing **Figure 1**.

DISCLOSURES:

The authors have no financial or personal relationship to disclose that would create a conflict of interest or bias.

REFERENCES:

1. Rudolph, A. M. & Heymann, M. A. The Circulation of the Fetus in Utero. *Circ Res* **21** (2), 163, doi:10.1161/01.RES.21.2.163 (1967).
2. Malik, A. B., Kaplan, J. E. & Saba, T. M. Reference sample method for cardiac output and regional blood flow determinations in the rat. *J Appl Physiol* **40** (3), 472 (1976).
3. Prinzen, F. W. & Bassingthwaighite, J. B. Blood flow distributions by microsphere deposition methods. *Cardiovasc Res* **45** (1), 13–21 (2000).
4. Van Oosterhout, M. F., Willigers, H. M., Reneman, R. S. & Prinzen, F. W. Fluorescent microspheres to measure organ perfusion: validation of a simplified sample processing technique. *Am J Physiol - Heart Circ Physiol* **269** (2), H725 (1995).
5. Glenny, R. W., Bernard, S. & Brinkley, M. Validation of fluorescent-labeled microspheres for measurement of regional organ perfusion. *J Appl Physiol* **4** (5), 2585 (1993).

6. Fujita, T. & Murakami, T. Microcirculation of monkey pancreas with special reference to the insulo-acinar portal system. A scanning electron microscope study of vascular casts. *Arch Histol Jpn Nihon Soshikigaku Kiroku* **35** (4), 255–263 (1973).
7. Bielke, S. R., Nagle, R. B., Trump, B. F. & Bulger, R. E. Scanning electron microscopy of renal vascular casts. *J Microsc* **108** (1), 89–96 (1976).
8. Dollinger, R. K. & Armstrong, P. B. Scanning electron microscopy of injection replicas of the chick embryo circulatory system. *J Microsc* **102** (2), 179–186, doi:10.1111/j.1365-2818.1974.tb03977.x (1974).
9. Murakami, T. Application of the scanning electron microscope to the study of the fine distribution of the blood vessels. *Arch Histol Jpn Nihon Soshikigaku Kiroku* **32** (5), 445–454 (1971).
10. Krucker, T., Lang, A. & Meyer, E. P. New polyurethane-based material for vascular corrosion casting with improved physical and imaging characteristics. *Microsc Res Tech* **69** (2), 138–147, doi:10.1002/jemt.20263 (2006).
11. Aharinejad, S., Franz, P., Böck, P., Lametschwandtner, A., Breiteneder, H. & Firbas, W. Sphincterlike structures in corrosion casts. *Scanning* **12** (5), 280–289, doi:10.1002/sca.4950120507 (1990).
12. Bosmia, A. N., Hogan, E., Loukas, M., Tubbs, R. S. & Cohen-Gadol, A. A. Blood supply to the human spinal cord: part I. Anatomy and hemodynamics. *Clin Anat (New York, N.Y.)* **28** (1), 52–64, doi:10.1002/ca.22281 (2015).
13. Kari, F. A. *et al.* New Insights into Spinal Cord Ischemia after Thoracic Aortic Procedures: The Importance of Anterior Radiculo-Medullary Artery Anatomy for Surgical Outcome. *Thorac Cardiovasc Surg* **65** (S 01), S1–S110, doi:10.1055/s-0037-1598779 (2017).
14. Griep, E. B. & Griep, R. B. The Collateral Network Concept: Minimizing Paraplegia Secondary to Thoracoabdominal Aortic Aneurysm Resection. *Tex Heart Inst J* **37** (6), 672–674 (2010).
15. Kari, F. A. *et al.* Immediate Spinal Cord Collateral Blood Flow During Thoracic Aortic Procedures: The Role of Epidural Arcades. *Semin Thorac Cardiovasc Surg* **28** (2), 378–387, doi:10.1053/j.semtcvs.2016.06.004
16. Kari, F. A. *et al.* Immediate Spinal Cord Backup Flow during Frozen Elephant Trunk Procedure: Intraspinal or Paraspinal System? *Thorac Cardiovasc Surg* **64** (S 01), OP174, doi:10.1055/s-0036-1571608 (2016).
17. Seldinger, S. I. Catheter replacement of the needle in percutaneous arteriography; a new technique. *Acta Radiol* **39** (5), 368–376 (1953).
18. Buckberg, G. D., Luck, J. C., Payne, D. B., Hoffman, J. I., Archie, J. P. & Fixler, D. E. Some sources of error in measuring regional blood flow with radioactive microspheres. *J Appl Physiol* **31** (4), 598–604 (1971).
19. Schimmel, C., Frazer, D. & Glenny, R. W. Extending fluorescent microsphere methods for regional organ blood flow to 13 simultaneous colors. *Am J Physiol - Heart Circ Physiol* **280** (6), H2496 (2001).
20. Powers, K. ., Schimmel, C., Glenny, R. . & Bernards, C. . Cerebral blood flow determinations using fluorescent microspheres: variations on the sedimentation method validated. *J Neurosci Methods* **87** (2), 159–165, doi:10.1016/S0165-0270(98)00180-0 (1999).
21. Meffert, P., Bischoff, M. S., Brenner, R., Siepe, M., Beyersdorf, F. & Kari, F. A. Significance

and function of different spinal collateral compartments following thoracic aortic surgery: immediate versus long-term flow compensation. *Eur J Cardiothorac Surg* **45** (5), 799–804, doi:10.1093/ejcts/ezt479 (2014).

22. Zulch, K. J. [Deficient circulation in the border zone of the two vascular regions as a cause of hitherto unexplained injuries of the spinal cord]. *Dtsch Z Nervenheilkd* **172** (1), 81–101 (1954).

23. Zülch, K. J. Pathogenetic and clinical observations in spinovascular insufficiency. *Zentralbl Neurochir* **37** (1), 1–13 (1976).

24. Cheshire, W. P., Santos, C. C., Massey, E. W. & Howard, J. F. Spinal cord infarction: etiology and outcome. *Neurology* **47** (2), 321–330 (1996).

25. Shamji, M. F., Maziak, D. E., Shamji, F. M., Ginsberg, R. J. & Pon, R. Circulation of the spinal cord: an important consideration for thoracic surgeons. *Ann Thorac Surg* **76** (1), 315–321 (2003).

26. Hickey, R., Albin, M. S., Bunegin, L. & Gelineau, J. Autoregulation of spinal cord blood flow: is the cord a microcosm of the brain? *Stroke* **17** (6), 1183–1189 (1986).

27. Gillilan, L. A. The arterial blood supply of the human spinal cord. *J Comp Neurol* **110** (1), 75–103 (1958).

28. Aminoff, M. J. *Neurology and General Medicine*. at <<https://books.google.de/books?id=IB6oVJ50oL4C>> (Churchill Livingstone: 2008).

29. Adamkiewicz, A. *Die Blutgefäße des menschlichen Rückenmarkes. I. Theil. Die Gefäße der Rückenmarkssubstanz. Sitzungsberichten der Kaiserlichen Akademie der Wissenschaften, Mathematisch-naturwissenschaftliche Classe.* **84**, 469–502 (1881).

30. Gailloud, P. The artery of von Haller: a constant anterior radiculomedullary artery at the upper thoracic level. *Neurosurgery* **73** (6), 1034–1043, doi:10.1227/NEU.0000000000000163 (2013).

31. Henson, R. A. & Parsons, M. Ischaemic lesions of the spinal cord: an illustrated review. *QJM: Int J Med* **36** (2), 205–222, doi:10.1093/oxfordjournals.qjmed.a067108 (1967).

32. Perk, J. Spinal Cord Diseases. *Encyclopedia of the Neurological Sciences (Second Edition)*, 264–268, doi:10.1016/B978-0-12-385157-4.00644-8 (2014).

33. Dommissie, G. F. The blood supply of the spinal cord. A critical vascular zone in spinal surgery. *J Bone Joint Surg Br* **56** (2), 225–235 (1974).

34. Suh, T. & Alexander, L. Vascular system of the human spinal cord. *Arch Neurol Psychiatry* **41**, 659–677 (1939).

35. Etz, C. D. *et al.* Spinal cord blood flow and ischemic injury after experimental sacrifice of thoracic and abdominal segmental arteries. *Eur J Cardio-Thorac Surg* **33** (6), 1030–1038, doi:10.1016/j.ejcts.2008.01.069 (2008).

36. Kieffer, E., Fukui, S., Chiras, J., Koskas, F., Bahnini, A. & Cormier, E. Spinal cord arteriography: A safe adjunct before descending thoracic or thoracoabdominal aortic aneurysmectomy. *J Vasc Surg* **35** (2), 262–268, doi:10.1067/mva.2002.120378

37. Williams, G. M., Roseborough, G. S., Webb, T. H., Perler, B. A. & Krosnick, T. Preoperative selective intercostal angiography in patients undergoing thoracoabdominal aneurysm repair. *J Vasc Surg* **39** (2), 314–321, doi:10.1016/j.jvs.2003.09.039

38. Kieffer, E., Fukui, S., Chiras, J., Koskas, F., Bahnini, A. & Cormier, E. Spinal cord arteriography: A safe adjunct before descending thoracic or thoracoabdominal aortic aneurysmectomy. *J Vasc Surg* **35** (2), 262–268, doi:10.1067/mva.2002.120378

39. Nijenhuis, R. J., Krings, T., Mull, M., Thron, A., Wilmink, J. T. & Backes, W. H. Non-invasive Spinal Cord Angiography for Imaging Vascular Spinal Cord Malformations. *Neuroradiol J* **22** (1_suppl), 107–114, doi:10.1177/197140090902205119 (2009).
40. Kawaharada, N. *et al.* Magnetic resonance angiographic localization of the artery of Adamkiewicz for spinal cord blood supply. *Ann Thorac Surg* **78** (3), 846-851; discussion 851-852, doi:10.1016/j.athoracsur.2004.02.085 (2004).
41. Hyodoh, H. *et al.* Usefulness of Preoperative Detection of Artery of Adamkiewicz with Dynamic Contrast-enhanced MR Angiography. *Radiology* **236** (3), 1004–1009, doi:10.1148/radiol.2363040911 (2005).
42. Backes, W. H., Nijenhuis, R. J., Mess, W. H., Wilmink, F. A., Schurink, G. W. H. & Jacobs, M. J. Magnetic resonance angiography of collateral blood supply to spinal cord in thoracic and thoracoabdominal aortic aneurysm patients. *J Vasc Surg* **48** (2), 261–271, doi:10.1016/j.jvs.2008.03.015

Figure 1 Schematic illustration

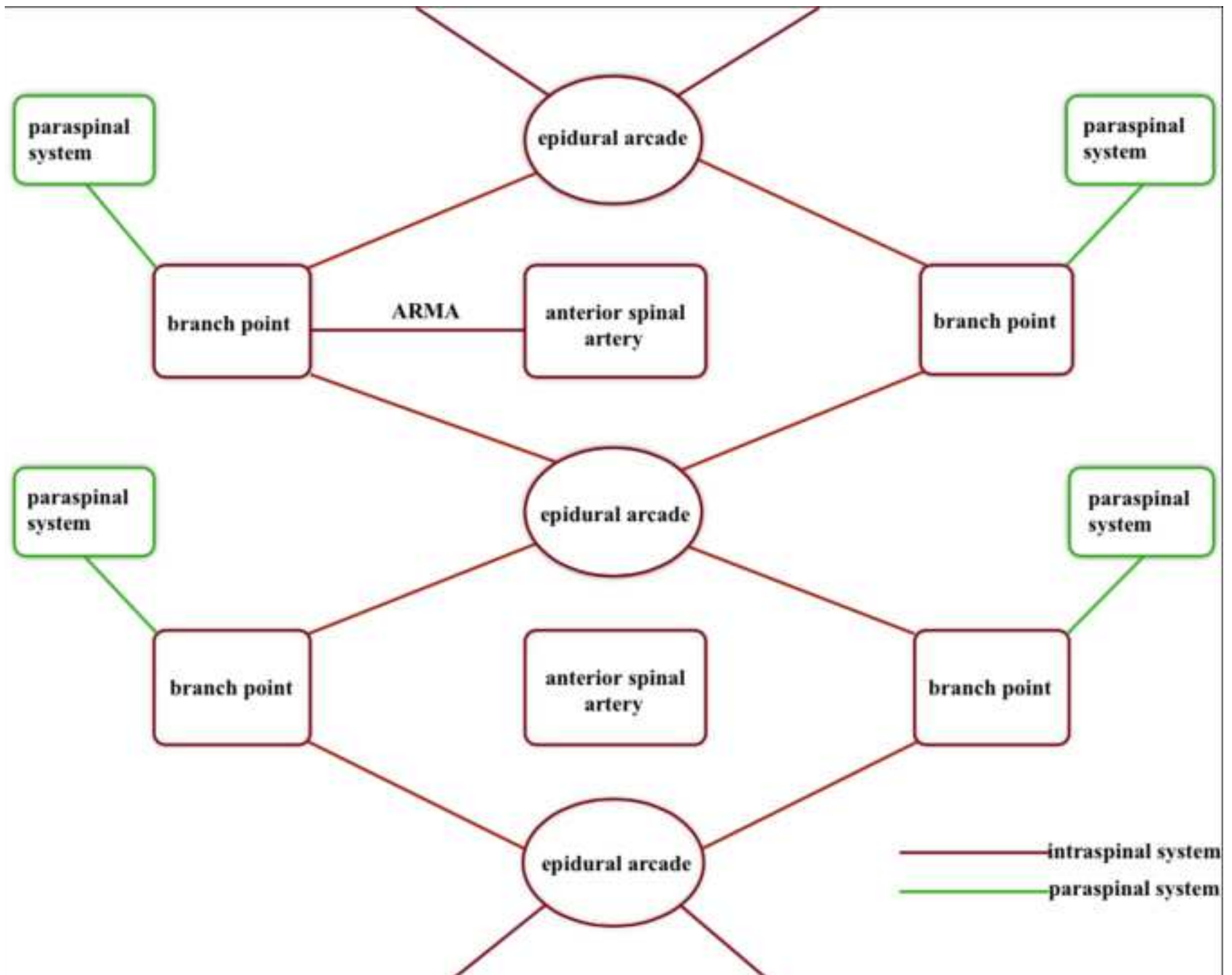


Figure 1 schematic illustration 2

[Click here to download Figure Figure 1 anatomical schematic illustration.tif](#)

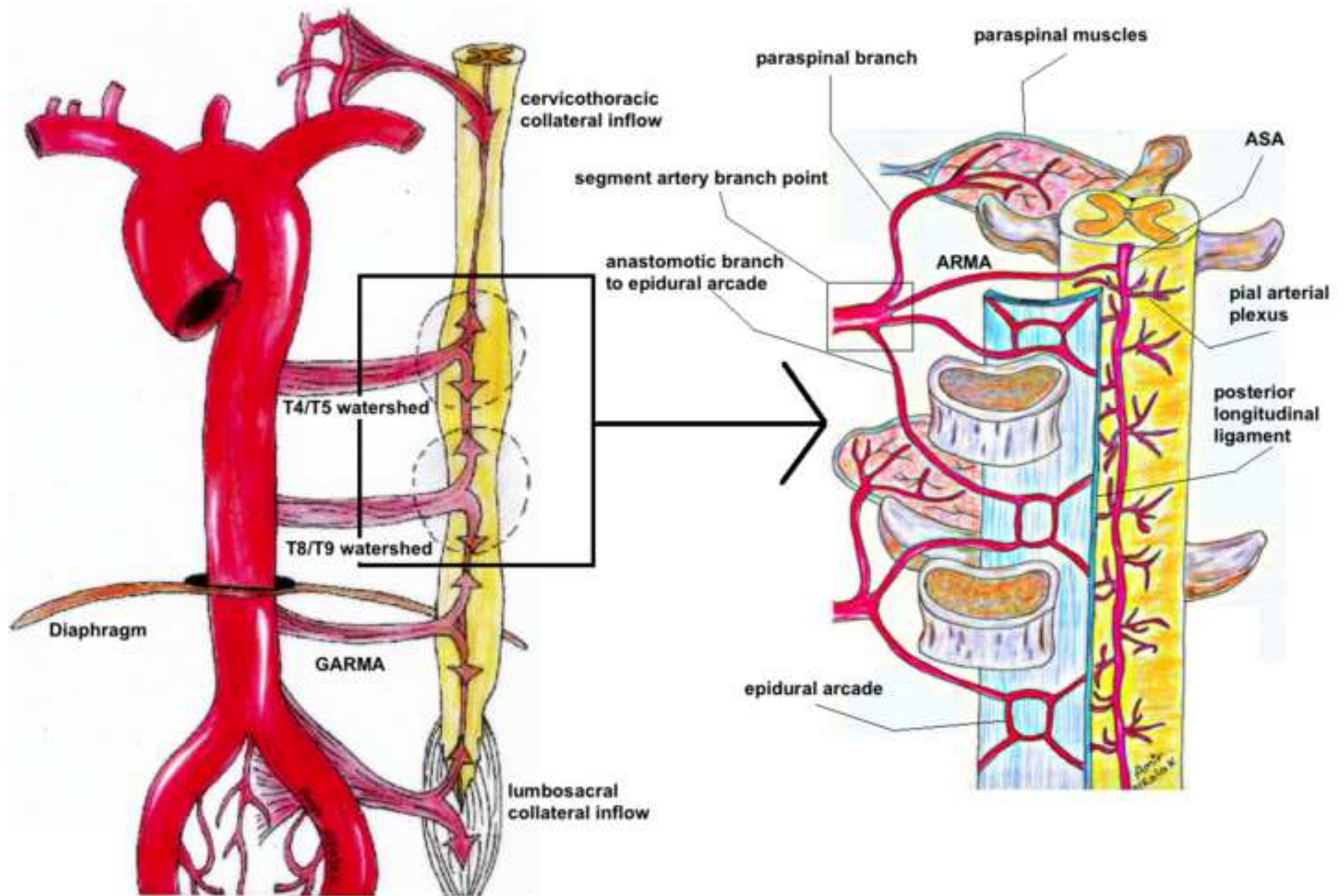


Figure 2 Spinal cord blood flow

[Click here to download Figure Figure 2 JOVE Fabian](#)

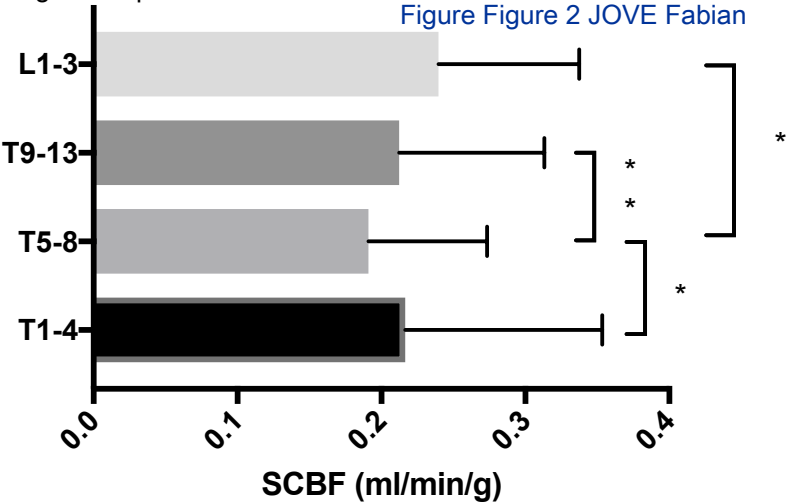


Figure 3. Spinal cord blood flow. Repeated

[Click here to download Figure Figure 3 JOVE](#)

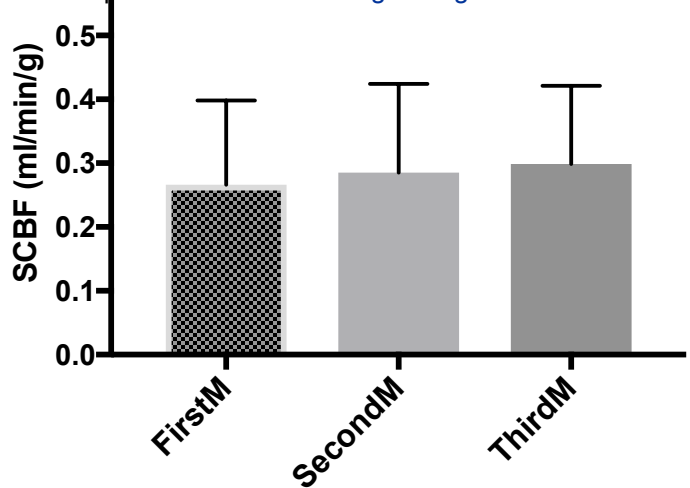
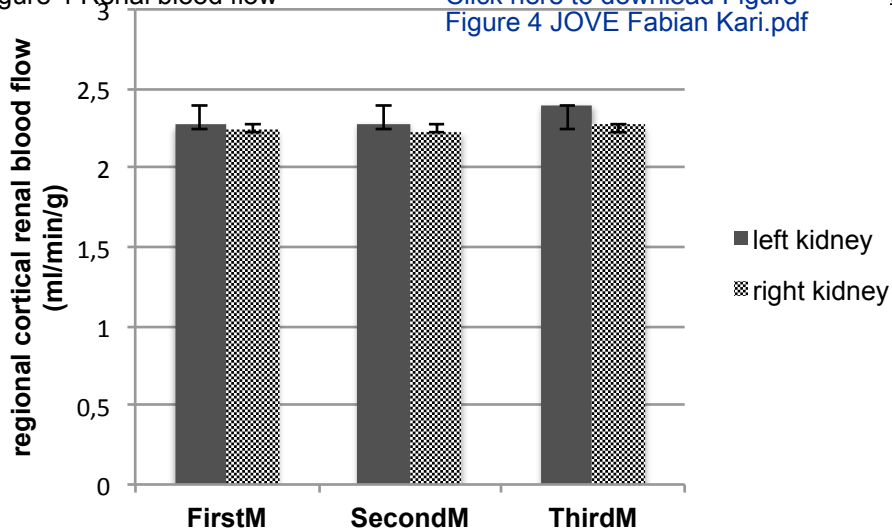


Figure 4 Renal blood flow

[Click here to download Figure 4 JOVE Fabian Kari.pdf](#)



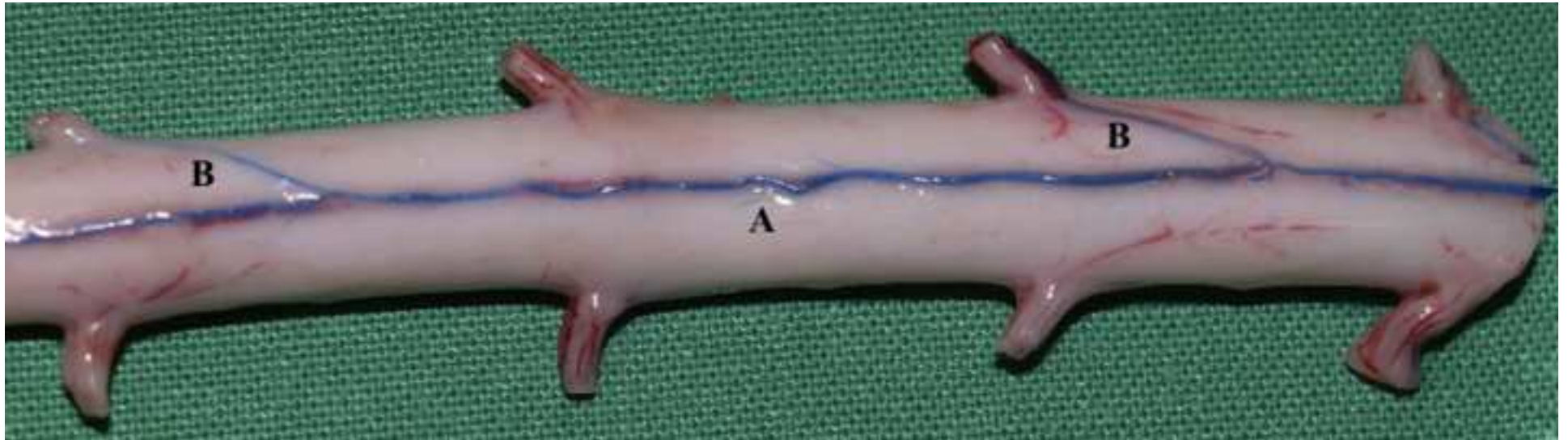


Figure 6 Casting ARMA

[Click here to download Figure
Figure 6 JOVE Fabian Kari.pdf](#)

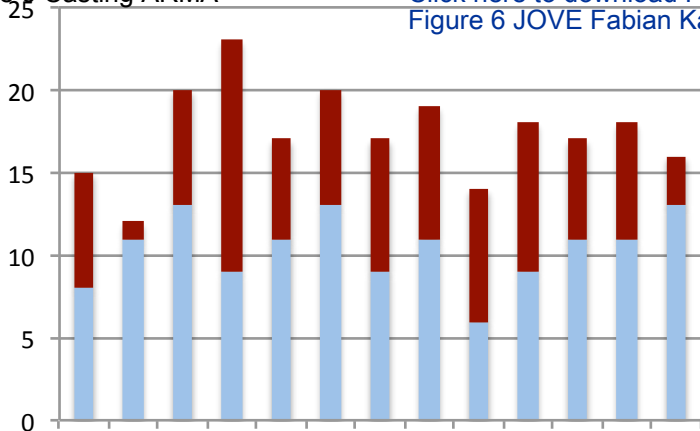


ARMA

RIGHT
LEFT

T1 T2 T3 T4 T5 T6 T7 T8 T9 T10 T11 T12 T13

VERTEBRAL SEGMENT



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Fluospheres Green	Molecular Probes	F21010	
Fluospheres Red	Molecular Probes	F8842	
Fluospheres Yellow	Molecular Probes	F21011	
Biofuge Primo R	Thermo Scientific		
Water Bath GFL Type 1083	GFL		
Weighting Scale	SCALTEC		
Fluorescence spektrometer LS55	Perkin Elmer		
PU4ii resin, hardener, pigment	vasQtec		blue pigment was used
15-ml-conical-tubes	TPP	91015	
Cellosolve acetate	Sigma-Aldrich	109967	
potassium hydroxide >85%	Carl Roth	P747.1	
Ethanol >99.5%	Carl Roth	5054.1	
Triton X-100	Sigma-Aldrich	X100	
Withdrawal pump 11	Harvard Apparatus		
3-way stopcocks	B. Braun	16496	
Perfusor lines PVC 150 cm/2.6 ml	B. Braun	8722960	
4-0 prolene suture	Ethicon	EH7411H	
		10ml, 20ml and	
Syringes	B. Braun	50ml	
Vasofix Braun 18G	B.Braun	4268130B	

Dormicum Midazolam 5mg/ml	Roche Pharma Medistar	3085793
Ketamin 10%	GmbH	3048734
Kaliumchlorid	B.Braun	3140598
tracheal tubus 6.5mm	Rüsch	112482
Fentanyl	Janssen- Cilag AstraZene	
Mepivacain Scandicain 2%	ca	910860
Microtome blade S35	Feather	207500000
disposable blade #11, #10	B.Braun	8080168
French-14-catheter	B.Braun	4476142
French-3-catheter	Vygon	115,094



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Author(s):

Item 1 (check one box): The Author elects to have the Materials be made available (as described at <http://www.jove.com/author>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
- ☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
- ☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JoVE**” means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JoVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's


expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Fabian A. Kari		
Department:	Cardiovascular Surgery		
Institution:	Heart Center Freiburg University		
Article Title:	Analysis of spinal cord blood supply combining vascular corrosion casting and fluorescence microsphere technique		
Signature:	 <div style="font-size: small;"> Digital unterschrieben von Fabian A. Kari DN: cn=Fabian A. Kari, o=UHZ Freiburg, ou=Herzchirurgie, email=fabian.alexander.kari@universitaet- s-herzzentrum.de, c=DE Datum: 2017.03.12 18:28:03 +01'00' </div>	Date:	12.03.2017

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Dear Editor,

We would like to thank you for the insightful comments and your help. We made the changes according to the comments.

Best Regards

Step 1.6: Please add details on how to dissect free the artery and vein. For e.g. using scalpel and scissors, etc. Please provide a reference for Seldinger technique. How is CVP and MAP measured?

Answer:

Thank you, we added details and the reference for this step. Changes made: page 3, lines 187-190.

2.1: Please add details on how to carry out thoracotomy. How is the incision made? JoVE veterinary reviewers typically recommend initial incisions be made with a scalpel rather than scissors.

Answer:

Thank you, we added details for this step. Changes made: page 3, lines 195-196.

2.2: Dissect how? Please add details and mention the instruments used.

Answer:

We added materials used for this step. Changes made: page 3, lines 200-201.

2.3: Open how? Please mention the type of catheter.

Answer:

We added details for this step. Changes made: page 3, lines 204-207.

3.2: What size catheter?

Answer:

Information was added. Changes made: page 4, line 270.

3.3: Please rewrite this step for clarity. Is it g or mL?

Answer:

Thank you, it is ml. We rewrote this step and added details. Changes made: page 5, lines 273-278.

3.4.1: Between which steps? Please add details on how to withdraw the casting material.

Answer:

We added the step. The withdrawal part was deleted for clarity. It is described in step 3.4.

Changes made: page 5, line 286.

Section 4: How long after injecting the casting material, is the autopsy performed?

Answer:

Thank you, information was added on page 5 lines 293-294.

4.1: How is the incision made? Please rewrite the second sentence for clarity.

Answer:

We rewrote this step and added details. Changes made: page 5, lines 297-298.

4.5: Please add more details.

Answer:

We added more details for this step. Changes made: page 6, lines 314-319.