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Fluorescence Angiography for Evaluation of Aneurysm Perfusion and Parent Artery Patency in Rodent Aneurysm Models: Technical Note

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Corresponding Author:	S Dr. Marbacher Kantonsspital Aarau Aarau, AG SWITZERLAND
Corresponding Author's Institution:	Kantonsspital Aarau
Corresponding Author E-Mail:	Serge.Marbacher@ksa.ch
Order of Authors:	Fabio Strange
	Sivani Sivanrupan
	Basil E. Grueter
	Jeanine Rey
	Dominik Taeschler
	Javier Fandino
	Serge Marbacher
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TITLE:

Fluorescence Angiography for Evaluation of Aneurysm Perfusion and Parent Artery Patency in Rat and Rabbit Aneurysm Models

AUTHORS, AFFILIATIONS:

Fabio Strange[1,2], Sivani Sivanrupan[2], Basil E. Grueter[1,2], Jeannine Rey[2], Dominik Taeschler[2], Javier Fandino[1,2], Serge Marbacher[1,2]

[1]Department of Neurosurgery, Kantonsspital Aarau, Switzerland
[2]Cerebrovascular Research Group, Department for BioMedical Research (DBMR), University of Bern, Bern, Switzerland

Corresponding author:

Fabio Strange (fabio.strange@ksa.ch; neurosurgery@ksa.ch)

Email address co-authors:

Sivani Sivanrupan (sivani.sivanrupan@students.unibe.ch)
Basil E. Grueter (basil.grueter@ksa.ch)
Jeannine Rey (jeanninerey@gmx.ch)
Dominik Taeschler (dominik.taeschler@gmx.ch)
Javier Fandino (javier.fandino@ksa.ch)
Serge Marbacher (serge.marbacher@ksa.ch)

KEYWORDS:

experimental aneurysm, rabbit, fluorescein, fluorescence, videoangiography, patency

SUMMARY:

We present a protocol to efficiently evaluate aneurysm perfusion and vessel patency of sidewall aneurysm in rats and rabbits, using fluorescein-based fluorescence video angiography (FVA). With a positive predictive value of 92.6%, it is a simple but very effective and economical method with no special equipment required.

ABSTRACT:

Brain aneurysm treatment focuses on achieving complete occlusion, as well as preserving blood flow in the parent artery. Fluorescein sodium and indocyanine green are used to enable the observation of blood flow and vessel perfusion status, respectively. The aim of this study is to apply FVA to verify real-time blood flow, vessel perfusion status and occlusion of aneurysms after induction of sidewall aneurysms in rabbits and rats, as well as to validate the procedure in these species.

Twenty sidewall aneurysms were created in 10 rabbits by suturing a decellularized arterial vessel pouch on the carotid artery of a donor rabbit. In addition, 48 microsurgical sidewall aneurysms were created in 48 rats. During follow-up at one month after creation, the parent artery/aneurysm complex was dissected and FVA was performed using an intravenous

fluorescein (10%, 1 mL) injection via an ear vein catheterization in rabbits and a femoral vein catherization in rats. Aneurysms were then harvested, and patency was evaluated macroscopically.

Macroscopically, 14 out of 16 aneurysms in rabbits indicated no residual parent artery perfusion with totally occluded luminae, however 11 (79%) were detected by FVA. Four aneurysms were excluded due to technical problems. In rats, residual aneurysm perfusion was macroscopically observed in 25 out of 48 cases. Of the 23 without macroscopic evidence of perfusion, FVA confirmed the incidence of 22 aneurysms (96%). There were no adverse events associated with FVA. Fluorescein is easily applicable and no special equipment is needed. It is a safe and extremely effective method for evaluating parent artery integrity and aneurysm patency/residual perfusion in an experimental setting with rabbits and rats. FVA using fluorescein as a contrast agent appears to be effective in controlling patency of aneurysms and the underlying vessel and can even be adapted to bypass surgery.

INTRODUCTION:

Evidence of complete aneurysm obliteration and parent artery integrity is of utmost importance in aneurysm surgery. There are several options to confirm parent artery patency and aneurysm occlusion, such as Doppler sonography, conventional cerebral angiography (DSA), computed tomography angiography (CTA) or magnetic resonance angiography (MRA)[1,2]. However, these are expensive and time-consuming methods which are often not available in a laboratory setting. Furthermore, they may have relevant side effects such as radiation exposure or need for additional sedation of experimental animals to avoid movement artefact.

With an increasing number of new endovascular devices emerging, there is a consecutive need for preclinical testing of such devices. However, these studies often rely on post-mortem analysis (e.g., macro pathology and histology) and lack information on dynamic perfusion. Furthermore, for the researcher it may be crucial to obtain immediate and reliable information during an experimental surgical procedure. Fluorescence angiography is a cost-effective and easy to perform visualization technique[1,3,4].

As such, indocyanine green (ICG) video angiography is often used in clinical neurosurgical procedures and has extensively been studied[5,6]. Fluorescein video angiography (FVA) is an alternative technique, with the additional advantage of creating a fluorescence signal that is within the wavelength range of human vision, and can thus be seen by the naked eye without an extended spectrum infrared camera[7]. Fluorescein video angiography is less often used in clinical cerebrovascular surgery and reports on FVA in experimental settings are scarce[1,4].

The aim of this report is to demonstrate the feasibility and scope of applications of FVA in rat and rabbit preclinical cerebrovascular research.

PROTOCOL:

The rodents were housed in an animal care facility and experiments were reviewed and approved by the Committee for Animal Welfare at the University of Bern, Switzerland (BE 108/16) and

(BE65/16). All animals were maintained on a standard laboratory diet with free access to food and water. All animal experiments were conducted under careful consideration of the 3Rs (replacement, reduction and refinement). Ten female New Zealand White rabbits and 48 male Wistar rats were included. ARRIVE guidelines were followed strictly[8].

NOTE: Twenty sidewall aneurysms were created in 10 rabbits by suturing a decellularized arterial vessel pouch on the carotid artery of a donor rabbit. In addition, 48 microsurgical sidewall aneurysms were created in 48 rats as described before[4,9]. The imaging procedure and macroscopic analysis was performed 4 weeks after aneurysm creation.

1. Preparation of material needed for fluorescein video angiography

1.1. Modify the flashlight by taping on a blue bandpass filter (see the **Table of Materials**), which will function as an excitation filter. The torch should then only emit blue light. Use black tape to avoid any leakage of unfiltered light.

1.2. Equip the camera (e.g., attached to the microscope) with a green bandpass filter (see the **Table of Materials**), which will function as a emission light filter. Only green light should now be able to pass through.

2. Preparation of workplace and materials

2.1. Disinfectant the workspace with alcohol.

2.2. Cover the table with sterile drapes to prevent contamination.

2.3. Use sterile instruments for the surgery.

3. Preparation of animals for the surgery

3.1. Weigh the animals.

3.2. Induce anaesthesia and adjust the dose according to the weight.

3.2.1. For **rabbits**, inject 30 mg/kg ketamine hydrochloride and 6 mg/kg xylazine intramuscularly. Shield their eyes with one hand during injection to reduce their fright reaction. Cover the cage with a sheet to help sedate the animals.

3.2.2. Anesthetize **rats** in a gas chamber with 4% isoflurane and 96% oxygen prior to the injection.

3.3. Monitor the depth of anaesthesia. Pinch between their toes to make sure the animals are asleep.

3.3.1. Reposition the **rabbits** onto their backs. They should not react.

3.3.2. For **rats**, pinch their tails and ensure that no reaction is observed.

3.4. Apply ointment on the rodents' eyes to prevent dryness. Pull out the rats' tongues to avoid any chance of swallowing.

3.5. Start with preservation of anaesthesia.

3.5.1. For **rabbits**, catheterize (22 G shielded IV catheter with injection port, see the **Table of Materials**) the ear vein. Inject xylazine (4 mg/kg/h), ketamine hydrochloride (40 mg/kg/h) and Ringer's Solution (500 mL/24 h) intravenously to preserve the anaesthesia. Use a three-way stopcock to enable multiple simultaneous injections.

3.5.2. For **rats**, inject 50 mg/kg ketamine hydrochloride and 0.5 mg/kg medetomidine hydrochloride intraperitoneally. Monitor anaesthesia with a noxious toe pinch during surgery. In the case of reaction, administer additional anaesthetic.

3.6. Tape the animals onto the board in a supine position and closely shave the incision location. Disinfect the area with alcohol.

3.6.1. For **rabbits**, disinfect the neck, especially around sternocleidomastoid muscle.

3.6.2. For **rats**, disinfect the area from bladder to transvers colon.

3.7. Administer oxygen through a mask throughout the surgery and maintain body temperature with a heating pad.

4. Preparation of the artery

4.1. For best results, thoroughly dissect the chosen vessel from the surrounding tissue[9,10].

4.1.1. For **rats**, identify the tail vein (less invasive, preferably used for surviving animals) or dissect a femoral vein for fluorescein injection[4,11].

NOTE: For **rabbits**, no further dissection of vessels is needed for fluorescein injection as the ear vein is already being used for anaesthesia.

4.2. Position a white pad under chosen vessel to increase contrast with the surrounding tissue.

4.3. Focus the camera mounted to the microscope on the dissected artery.

5. Fluorescein video angiography

5.1. Cover the 5 mL syringe filled with fluorescein sodium (100 mg/mL, see the **Table of Materials**) with aluminium foil to protect from exposure of light.

Turn off the lights (as much as possible) and inject fluorescein sodium intravenously. Inject under darkness to prevent photobleaching.

5.1.1. For **rabbits**, inject 0.3 mL/kg fluorescein sodium through the three-way-stopcock into the catheterized ear vein.

5.1.2. For **rats**, inject 0.4 mL/kg fluorescein sodium into the femoral vein via a catheter or a 25 G needle.

5.2. Flush the needle or the catheter with 0.5 mL saline solution to ensure that all dye is injected.

5.3. Illuminate the surgical field with the modified flashlight.

5.4. Commence filming with the modified camera. Blood flow should be visible a few seconds after injection (**Figure 1**).

NOTE: Here, we used frame rate = 50 frames/s, focal length = 70 mm, and F3.4.

6. Macroscopic analysis

6.1. Resect the aneurysms and parent artery complex, and evaluate the patency macroscopically by opening the parent artery with micro-scissors and evaluate the lumen of the parent artery and the anerysm's orifice (see **Figure 1,2**)[9]. Measure the sizes of the aneurysms. Aneurysm-parent-artery-complex can then be stored for further analysis (e.g., histology).

RESULTS:

Heart rate and blood pressure were monitored during surgery. Mean heart rate was 193/min in rabbits and 196/min in rats. The rabbits' body weight ranged 3.05–4.18 kg, and the rats weighed 335–690 g.

We were able to perform FVA in eight out of ten rabbits (**Figure 1**). Four aneurysm examinations in two rabbits were not recorded with the camera due to technical difficulties. No technical difficulties involving FVA in rats were reported. However, FVA could not be performed in one rat due to difficulties puncturing the femoral vein.

Of 16 aneurysms in eight rabbits, two aneurysms showed persistent perfusion of the parent artery (confirmed macroscopically) (see **Table 1**) while FVA identified five cases with residual perfusion. 14 rabbit aneurysms showed no residual perfusion macroscopically, however 11 (79%) were subsequently detected using FVA. Residual perfusion was observed macroscopically in 25 of 48 rats (**Table 1**), and the other 23 rats showed no macroscopic signs of residual perfusion (**Figure 2**). 22 of those 23 aneurysms were then confirmed using FVA (96%). Altogether, 25 of 27

cases could be confirmed, resulting in a positive predictive value of 92.6%, a sensitivity rate of 100% and specificity of 94.1%. (**Table 2**).

In summary, 25 aneurysms showed residual perfusion, 53 parent arteries were patent and 11 were occluded as confirmed macroscopically and on video angiography. There were only minor complications associated with FVA in rabbits; such as perforation of marginal ear vein during catheterization. No further adverse events were experienced. No mortality and no morbidity due to FVA was reported.

FIGURE AND TABLE LEGENDS:

Figure 1: Visualizing patency in a rabbit. (**A**) Patency of the parent artery is clearly visible on the fluorescence image (green emission from fluorescein are seen). (**B**) This artery is occluded (fluorescence image). Both arteries were inspected macroscopically (**C**–**D**). Panel (**C**) shows in the artery from panel A; the lumen is open. Panel (**D**) shows artery from panel B where occlusion can be seen macroscopically. Orange dotted lines mark the borders of the parent artery.

Figure 2: Visualizing perfusion in a rat. (**A**) This panel shows a residually perfused aneurysm (red dotted line marks the residual perfusion). (**B**) No perfusion can be detected. Panel (**C**) shows the artery from panel A during macroscopic scrutiny; the aneurysm orifice is open. (**D**) Macroscopic view of the neointima on an occluded aneurysm. Orange dotted lines mark the parent artery and the aneurysm' dome. Panels (A) and (B) are fluorescence-only images and the green color shows fluorescein emission.

Table 1: Patency testing. Patency of parent artery was only tested in rabbits and is illustrated here. Fluorescein detected more patencies of parent arteries than macroscopical evaluation. (All rats in this setting had an open parent artery, as aneurysms were sutured on the abdominal aorta.) The patency of aneurysms was tested in rats only. Twenty-two of 23 macroscopically detected patencies were confirmed using FVA. Twenty-one of 25 showed no patency on FVA.

Table 2: Two-by-two table used to calculate specificity and sensitivity of FVA.

DISCUSSION:

FVA is a promising and uncomplicated method to examine vessels in rodents and can be performed with commercial devices and off-the-shelf equipment. FVA can be implemented during any surgery where intraoperative evaluation of vessel integrity is needed as the vessels need proper dissection first.

The authors preferred venous injection to arterial injection due to the lower risk of inadvertent events such as infection, ischemia and compartment syndrome[12]. Intravenous injection enables dependable, spatially limited, highly concentrated staining, and requires small dye dosages[13,14]. Additionally, venous injection allows a quick clearance of fluorescein[14,15]. An alternative method is to inject contrast agent directly into the chosen artery. This method was not used in these experiments as the investigators wanted to prevent contaminating the surgical field with blood

and fluorescein. In order to reduce this risk, peripheral venous contrast agent injection is recommended[13].

Advantages of FVA are high contrast (easily detectable with the human eye), high sensitivity as shown above (**Table 2**), low cost and easy handling[16]. Fluorescein sodium was the chosen contrast agent to examine perfusion. Visible light alone can be used for excitation of the dye and emission of the typical green light. Nevertheless, this contrast agent works best with blue light (approximately 480 nm) and emits a strong green light (wavelength approximately 530 nm)[15]. According to Yoshioka et al., fluorescein colors the artery extremely quickly[14]. Furthermore, the flow of fluorescein-enriched blood can be observed in real time[15,17]. The short time needed for FVA presents another advantage; in this series it took an average of 2 min (± 1min) to conduct one FVA.

The disadvantage of using fluorescein as a contrast agent is that it works well with only thin artery walls which demand very careful dissection. Ichikawa et al. showed the extinction of dye due to the thwarted emission of light through thicker walls by calcification or undissected arteries[15]. After injection, fluorescein is metabolised to fluorescent fluorescein glucuronide in the liver. Within 30 min after injection, the concentration of fluorescein glucuronide exceeds the concentration of fluorescein[18]. Fluorescein requires a long clearance time. An immediate reinjection after intravenous injection of fluorescein is not recommended as the artery and aneurysm are already fluorescent from the first injection[17].

The molecular weight of fluorescein is only 376 kDa which allows leakage of the dye. The vascular wall also becomes fluorescent which could lead to false positive flow evaluations (increasing with time after application). A patchy coloration of the vessel wall was observed starting approximately 5 min after injection of fluorescein[14]. The spotty coloration, however, was only observed in larger arteries. Small and medium arteries did not show this staining structure[17]. It is recommended to evaluate the aneurysm immediately in order to detect residual filling.

Although there is a very low risk of toxicity, some cases of fluorescein leading to cardiac and respiratory reactions have been described[14]. In this study no severe adverse event occurred; the only complications were 2 cases of ear vein perforation. According to Lane et al., sodium fluorescein is not harmful even when used in humans[17]. On the other hand, fluorescein is quite unstable and should not be exposed to white light[16]—a red light source can be used instead.

In order to choose the concentration of fluorescein for rabbits, the investigators started with the lowest known working dose in rats (0.2 mL of 100 mg/mL fluorescein sodium) and increased it gradually to 1 mL. A strong fluorescence signal was registered at that dose. The dosage was increased gradually to test if the fluorescence improves—which was not the case. The authors decided to continue with 1 mL of 100 mg/mL fluorescein sodium[13].

Another dye available to examine vessels intraoperatively is ICG. Its size is 775 kDa and such barely penetrates the surrounding tissues[14]. Because of its longer emission wavelengths, tissue is penetrated more easily because tissues are more transparent at 800 nm[19] and deeper

structures become visible[14,16]. Excitation wavelength within the 750–800 nm are required[16,20] and the emission wavelength from the contrast agent is approximately 800 nm[16], making both invisible to the human eye. Due to its short half-life time in blood plasma, the dye can be injected and reused repeatedly[16]. Limitations to using this dye include problems with thick-walled arteries[20] and the necessity of light outside the visible spectrum[13]. As a consequence, ICG is dependent on expensive equipment and not readily applicable in every laboratory.

In conclusion, FVA is a fast, inexpensive and reliable method with high sensitivity to screen patency of aneurysms and parent arteries in rodent aneurysm models. It is associated with virtually no morbidity and mortality. It allows real-time blood flow monitoring during surgery and follow-up. To improve its efficacy, the injection should be carried out in the dark and is best performed on meticulously dissected vessels. This method can easily and safely be implemented in a cerebrovascular laboratory, and can minimize experiment costs.

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DISCLOSURE:

All authors confirm no conflicts of interest.

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Figure 1

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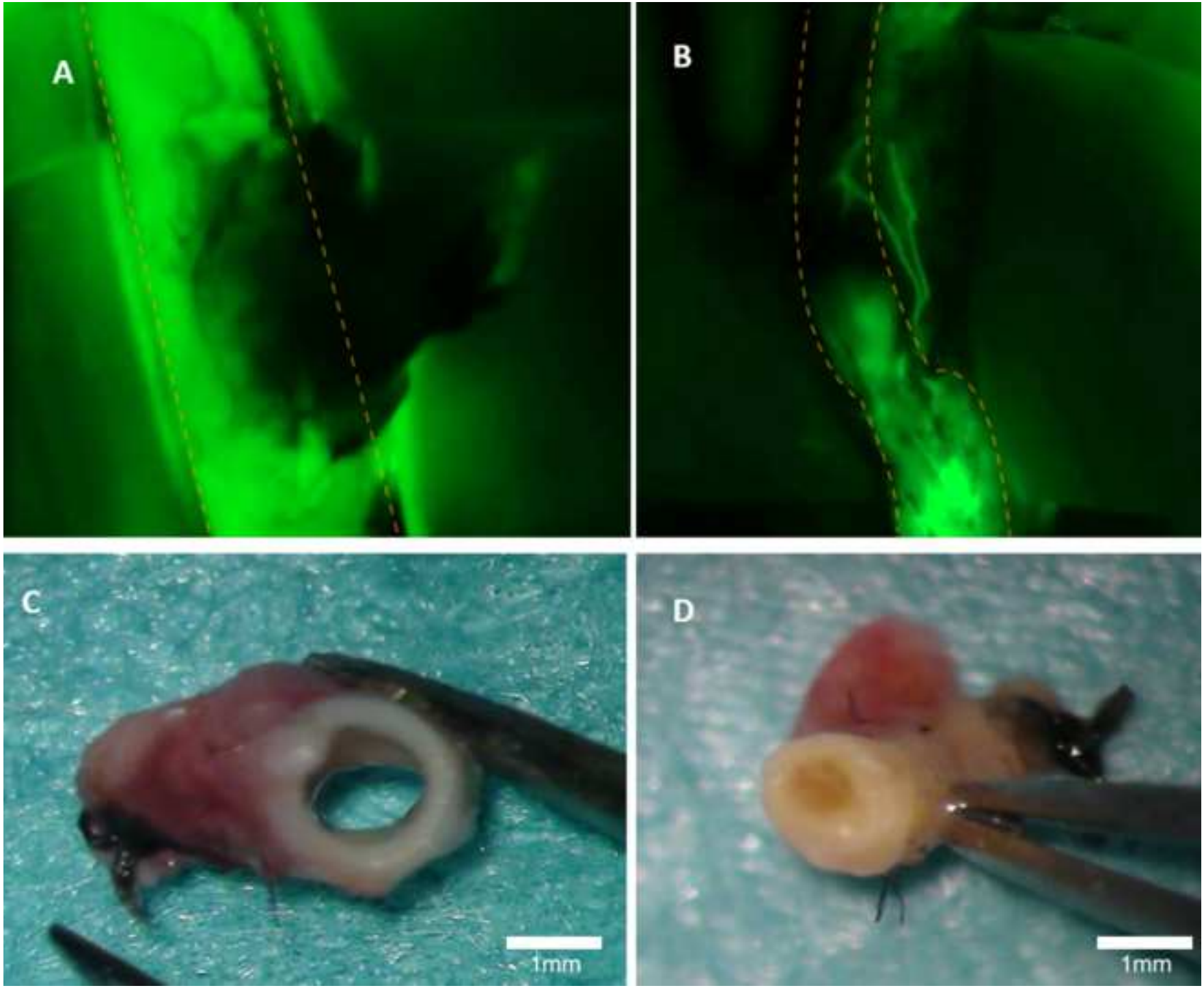

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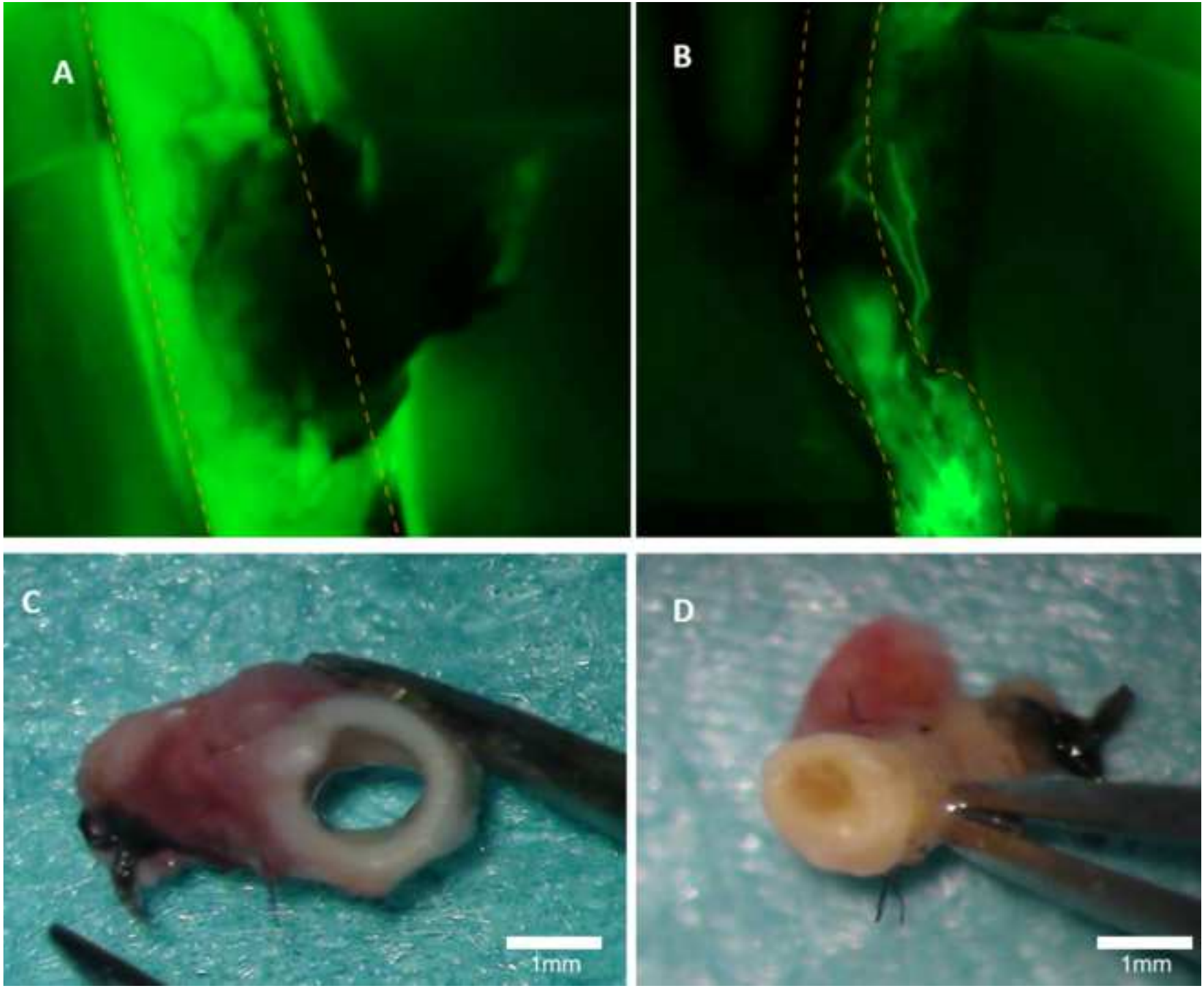

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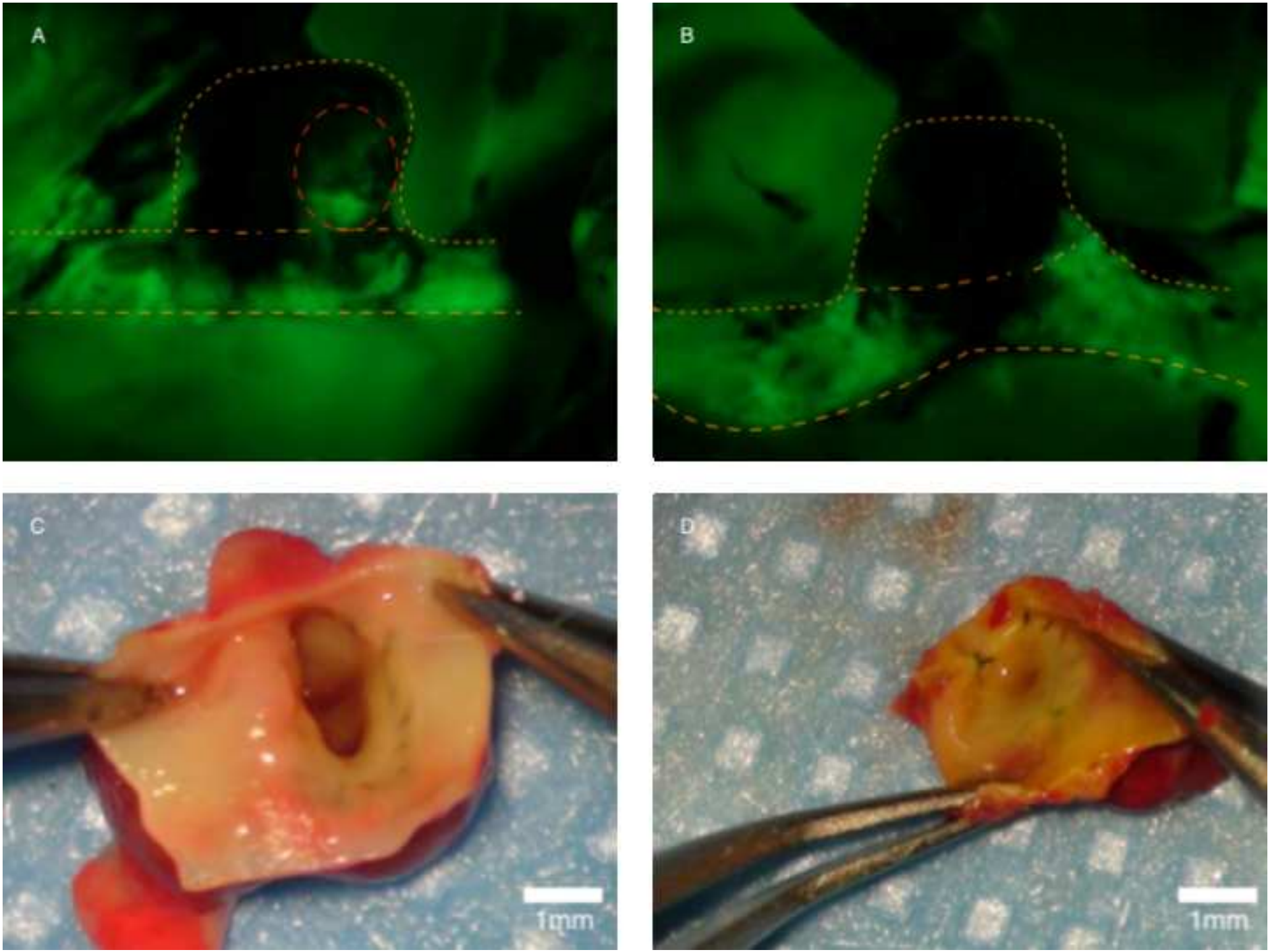

Figure 2

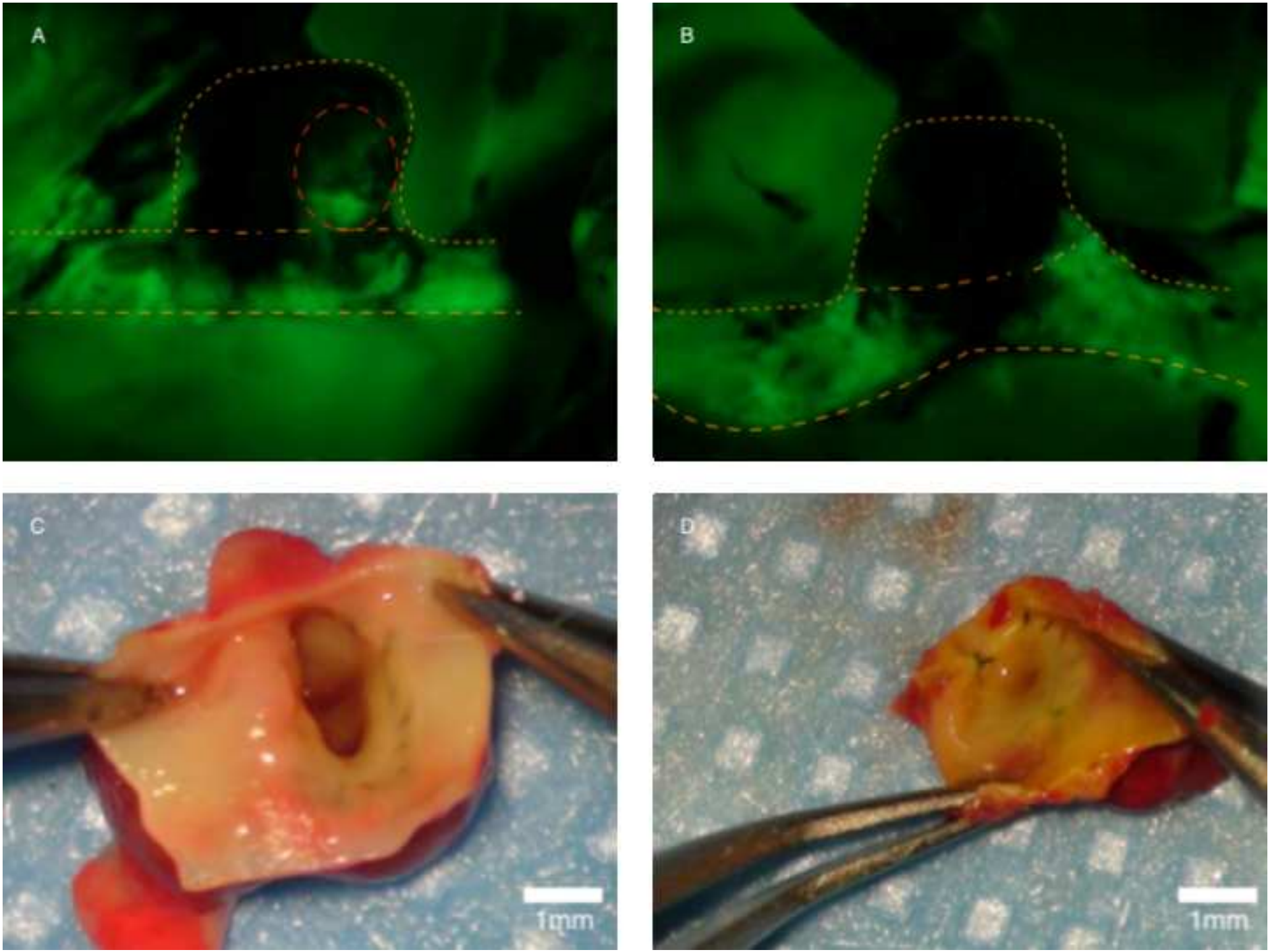

Table 2

	Macroscopic positive	Macroscopic negative	total
Fluorescein positive	25	2	27
Fluorescein negative	0	32	32
total	25	34	

Table 1

	Patency/Residual Perfusion			
	Macroscopical +	Macroscopical -	Fluorescein +	Fluorescein -
Rabbits	2	14	5	11
Rats	23	25	22	21
Total	25	39	27	32

Table 1

	Patency/Residual Perfusion			
	Macroscopical +	Macroscopical -	Fluorescein +	Fluorescein -
Rabbits	2	14	5	11
Rats	23	25	22	21
Total	25	39	27	32

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Aluminium foil			
Animal shaver			
Black tape			
Blue filter	Thorlabs MF475-35		
Body warm plate			
Camera	Sony NEX-5R		
Disinfictant			
	Fluorescein Faure		
Fluorescein sodium	10%		
Green filter	Thorlabs MF539-43		
Incontinence pad			
Isoflurane			
Ketamine hydrochloride			any generic products
Medetomidine hydrochloride			any generic products
Needle	25G		
Oxygen			
Plate			
Ringer's Solution			
Sterile sheets			
Surgical instruments			micro forceps, micro scissor, blunt surgical scisso
Surgical microscope	OPMI, Carl Zeiss AG, Oberkochen, Germany		
Syringe 2ml, 5ml			
Tape			
Torch light			

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Aluminium foil			
Animal shaver			
Black tape			
Blue filter	Thorlabs MF475-35		
Body warm plate			
Camera	Sony NEX-5R		
Catheter	22G Vasofix Safety		
Disinfictant			
Fluorescein sodium	Fluorescein Faure 10%		
Glas plate			
Green filter	Thorlabs MF539-43		
Incontinence pad			
Infusion pump	Perfusor Secura		
Ketamine hydrochloride			any generic products
Needle	25G		
Oxygen			
Ringer's Solution			
Sterile sheets			
Surgical instruments			micro forceps, micro scissor, blunt surgical scissor
Surgical microscope	OPMI, Carl Zeiss AG, Oberkochen, Germany		
Syringe 2ml, 5ml, 50ml			
Tape			
Three-way-stopcock			
Torch light			
Xylazin			any generic products

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CORRESPONDING AUTHOR

Name:	Strange Fabio
Department:	Department of Neurosurgery
Institution:	Kantonsspital Aarau, Switzerland
Title:	MD
Signature:	
Date:	February 1st 2018

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AUTHOR'S REVISION LETTER

We thank the editorial office for their comments and constructive criticisms that gave us the chance to improve our manuscript. We addressed these issues, revised the manuscript accordingly, and hope that these revisions ensure the suitability of our paper for publication. Please find a point-by-point response to the editorial office' comments below.

Technically rabbits are not rodents; please edit the title accordingly and avoid titles that are similar to your previous publcations.

Thank you, we changed the Title to "Fluorescence Angiography for Evaluation of Aneurysm Perfusion and Parent Artery Patency in a Rat and Rabbit Aneurysm Model
"

Please add this as a note in the protocol section. Please also cite references for how to create the aneurysms.

"Twenty sidewall aneurysms were created in 10 rabbits by suturing a decellularized arterial vessel pouch on the carotid artery of a donor rabbit. In addition, 48 microsurgical sidewall aneurysms were created in 48 rats."

Thank you, we added this section to the Protocol and cited accordingly.

These items are missing from the protocol. Please add: *Aneurysms were then harvested, and patency was evaluated macroscopically.*

Thank you, for this important point, we added this section to the Protocol as follows:

6. "Macroscopic Analysation

6.1. Aneurysms and parent artery complex are then to be explanted, and patency can be evaluated macroscopically by opening the parent artery and with micro scissors and evaluate the lumen of the parent artery and the anerysm's orifice (see Figure 1&2). The sizes of the aneurysms are to be measured.

6.2. Aneurysm-parent-artery-complex can then be stored for further analysation (eg. histology)"

How was residual artery perfusion tested?

The aneurysms/parent arteries without residual perfusion showed macroscopically total occlusion of the artery (see Figure D). The following addition was made to the manuscript:
"Macroscopically, 14 out of 16 aneurysms in rabbits indicated no residual parent artery perfusion with totally occluded luminae, however 11 (79%) were detected by FVA"

Because of the high tissue autofluorescence in this wavelength window. This point should be acknowledged in this manuscript.

Thank you for pointing this out, in this experimental setting, autofluorescence posed no distraction. Due to the white pad underneath the chosen vessel, the bandpass filter and the rapidly and strongly fluorescenting perfused vessels, autofluorescence was not detected by the camera

Please add information about aneurysm generation in a note here. Please also cite references for how to create the aneurysms. How long after aneurym creation is imaging performed?

Thank you, we added and cited accordingly: *"Twenty sidewall aneurysms were created in 10 rabbits by suturing a decellularized arterial vessel pouch on the carotid artery of a donor rabbit. In addition, 48 microsurgical sidewall aneurysms were created in 48 rats as described before."* Aneurysms where created 4 weeks prior to the imaging.

Mention catheter specifications?

We added the following detail to the manuscript:" *(22G Shielded IV catheter with injection port, see Table of Materials Rabbits)"*

When and how was the anuerysm generated?

Thank you for this comment, we added, as described above, a section to the Protocol stating the time (4 weeks prior to imaging) and cited the detailed procedure of the sidewall aneurysm creation.

Unclear which of the steps within this section describe artery preparation. The aneurysm creating and careful artery dissection appears to be missing from your protocol and appears to be one of the most important steps!

Than you, we added this information as follows:
Creating: line 94
Dissection line 162, 4.1
For details we added appropriate citations.

Unclear how this is done. Mention surgical tools used. Cite a reference.

Thank you, we added 2 refrences to this step how to dissect the femoral vein in rats:

Shurey S, Akelina Y, Legagneux J, Malzone G, Jiga L, Ghanem AM: The rat model in microsurgery education: classical exercises and new horizons. **Arch Plast Surg 41:**201-208, 2014

Gruter BE, Taschler D, Rey J, Strange F, Nevzati E, Fandino J, et al: Fluorescence Video Angiography for Evaluation of Dynamic Perfusion Status in an Aneurysm Preclinical Experimental Setting. **Oper Neurosurg (Hagerstown)**, 2019

What is the location of the aneurysm? How was it identified? How was it exposed?

Thank you for pointing this out, goal is to describe the FVA in any aneurysm or bypass model, therefore we now made it clearer by writing "chosen vessel" instead of "aneurysm".

When exactly was the artery dissected? Some steps are missing.

Than you, artery dissection is the first mandatory step when exposing the chosen vessel we mention this in 4.1

It is cannulated/catherized? Mention specifications of the items used if so. Mention needle gauge.

Thank you, indeed this was missing, we added the following addition: "*...fluorescein sodium into the femoral vein via a catheter or a 25G needle."*

Which pipe? How much saline?
Thank you, this was unclear, we added the following addition; *"...Flush the needle or the catheter with 0.5 ml saline..."*

What is the frame rate? Presumably you need to capture within the first few seconds or else the fluorescein will be taken up by the surrounding tissues and obscure the signal. Mention the lens focal length, aperture settings, F#.

Yes, thank you, this was missing; the framerate was 50 FPS, focal length 70mm. F 3.4. Yes, as mentioned the fluorescent will be visible in seconds and the uptake by the surrounding tissue takes longer but eventually yes, the signal will be partly obscured, still the vessels emit the most intense. Although, as described before, its important to place a white pad under the chosen vessel to increase contrast. (4.2)

Unclear how and when were the aneurysms produced in rats and rabbits? How many aneurysms per animal?
Thank you, indeed this was missing, we added this information as described before starting line 94

When was the puncture performed? This is missing from the protcol.
Thank you, we describe this in 5.2.2.

Unclear what is meant by residual perfusion in this context.
Thank you, indeed this sentence was misleading, we revised it to clearify; *"Of 16 aneurysms in eight rabbits, two aneurysms showed persistent perfusion of the parent artery (confirmed macroscopically) (see Table 1) while FVA identified five cases with residual perfusion."*

It is unclear what is meant by macroscopic evaluation of perfusion, please define it clear and acite a reference if needed.
Thank you, indeed, this was missing and unclear, we added therefore *6. Macroscopic Analysation* to the protocol

What are these macroscopic signs of perfusion? Please describe.
Please see above (6*. Macroscopic Analysation)*

Came back positive from where? Unclear what is being said here. Do you perform pathological evaluation?
Thank you, indeed, this wording was misleading, we redicated this as follows; *"...25 of 27 cases could be confirmed..."*

It is unclear how patency was tested, please describe. The protocol is missing this.
Yes, indeed, as described above we added "Macroscopic Analysation" to the Protocol

Macro pathology?
Yes, thank you, we describe this now in 6. "Macroscopic Analysation"

Please mention that this is a fluorescene-only image and that the green color showsn only fluorescein emission.

Thank you, we added this comment to mark A and B are fluorescence images only and the green color shows fluorescein emission.

There are no panel labels or redor orange markings on the figure. Please update.

Thank you, we updated the images with red and orange dotted lines

Please add a figure title. E.g. Visualizing perfusion

We added figure titles as follows:
"Figure 1: Visualizing patency in rabbit"
"Figure 2: Visualizing perfusion in rat"

Table mentions "residual perfusion", how was patency derived from this?

Thank you for pointing that out, we added "Patency" to the table

Unclear. Please revise this sentence. I think you mean that the vessel walls are too thick to allow emission light transmission through

Thank you, we totally agree and clarified the sentence as follows: *„Ichikawa et al. showed the extinction of dye due to the thwarted emission of light through thicker walls by calcification or undissected arteries "*