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TITLE:

Modeling Stroke in Mice: Transient Middle Cerebral Artery Occlusion Via the External Carotid Artery

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KEYWORDS:

stroke, brain ischemia, animal model, middle cerebral artery, transient

SUMMARY:

Different models of middle cerebral artery occlusion (MCAo) are used in experimental stroke research. Here, an experimental stroke model of transient MCAo via the external carotid artery (ECA) is described, which aims to mimic human stroke, in which the cerebrovascular thrombus is removed due to spontaneous clot lysis or therapy.

ABSTRACT:

Stroke is the third most common cause of mortality and the leading cause of acquired adult disability in developed countries. To date, therapeutic options are limited to a small proportion of stroke patients within the first hours after stroke. Novel therapeutic strategies are extensively being investigated, especially to prolong the therapeutic time window. These current investigations include the study of important pathophysiological pathways after stroke, such as post-stroke inflammation, angiogenesis, neuronal plasticity, and regeneration. Over the last decade, there is a growing concern about the low reproducibility of experimental results and scientific findings among independent research groups. To overcome the so-called “replication crisis”, detailed standardized models for all procedures are urgently needed. As an effort within the “ImmunoStroke” research consortium (https://immunostroke.de/), a standardized transient middle cerebral artery occlusion (MCAo) mouse model is proposed. This model allows the complete restoration of the blood flow when removing the filament, simulating the therapeutic or spontaneous clot lysis that occurs in a large proportion of human strokes. The surgical method of this “filament” stroke model and functional analysis tools are demonstrated in the accompanying video.

INTRODUCTION:

Stroke is one of the most common causes of death and disability worldwide. Although there are mainly two distinct forms of stroke, ischemic and hemorrhagic, 80–85% of all stroke cases are ischemic[1]. Only two treatments are currently available for ischemic stroke patients: pharmacological treatment with recombinant tissue plasminogen activator (rtPA) or mechanical thrombectomy. However, due to the narrow therapeutic time window and multiple exclusion criteria, only a select number of patients can benefit from these specific treatment options. Over the last two decades, preclinical and translational stroke research has been centered on the study of neuroprotective approaches. However, all compounds that reached clinical trials have so far shown no improvements for the patient[2].

As an in vitro model cannot accurately model brain interactions and pathophysiological mechanisms before and after stroke, animal models are crucial for preclinical stroke research. Mimicking all aspects of human ischemic stroke in a single animal model is not feasible as ischemic stroke is a complex and heterogeneous disease. For this reason, different ischemic stroke models have been developed in different species. Brain ischemia due to photothrombosis of cerebral arterioles or by permanent distal occlusion of the middle cerebral artery (MCA) are commonly used models that induce small and locally defined lesions in the neocortex[3,4]. However, the probably most commonly used stroke model is the so-called “filament model,” in which a transient MCA occlusion is achieved. This model consists of a transient introduction of a suture filament until the origin of the MCA, through the internal carotid artery, resulting in a sharp reduction of the cerebral blood flow and the subsequent large infarction of subcortical and cortical brain regions[5].

Although most stroke models mimic occlusions of the MCA[6], the "filament model" enables precise delimitation of the ischemic interval depending on the reperfusion time point. Reperfusion by filament removal mimics the human clinical scenario of restored cerebral blood flow after spontaneous or therapeutic (rtPA or mechanical thrombectomy) clot lysis. Different modifications of this “filament model” have been described to date. In the most common approach, first described by Longa *et al*. in 1989[5], a silicon-coated filament is introduced via the common carotid artery (CCA) and advanced through the internal carotid artery (ICA) into the Circle of Willis, where it blocks the origin of the MCA[7]. Although a very commonly used approach, this model does not allow the complete restoration of the blood flow during the reperfusion as the CCA is permanently ligated after removing the filament.

Over the past decade, an increasing number of research groups have been interested in modeling stroke in mice using the “filament model.” However, the considerable variability of this model and the lack of standardization of the procedures are some of the reasons for the high variability and poor reproducibility of the experimental results and scientific findings reported so far[2,8]. A potential cause of the current “replication crisis,” referring to the low reproducibility among research laboratories, is the non-comparable stroke infarct volumes between research groups using the same experimental methodology[9]. Indeed, after conducting the first preclinical randomized controlled multicenter trial study[10], we could confirm that the lack of sufficient standardization of this experimental stroke model and the subsequent outcome parameters

were the main reasons for failing reproducibility in preclinical studies between independent laboratories[11]. Such drastic differences in the resulting infarct sizes, despite the use of the same stroke model, justifiably pose not only a threat to confirmatory research, but also for scientific collaborations due to the lack of robust and reproducible models.

In light of these challenges, we aimed to develop and describe in detail the procedure for a standardized transient MCAo model as it is used for the collaborative research efforts within the "ImmunoStroke" research consortium (https://immunostroke.de/). This consortium aims to understand the brain-immune interactions underlying the mechanistic principles of stroke recovery. Additionally, histological and related functional methods for the analysis of stroke outcome are presented. All methods are based on standard operating procedures established and used in all research laboratories within the ImmunoStroke consortium.

PROTOCOL:

The experiments reported in this video were conducted following the national guidelines for the use of experimental animals, and the protocols were approved by the German governmental committees (Regierung von Oberbayern, Munich, Germany). Ten-week-old male C57Bl/6J mice were used. The animals were housed under controlled temperature (22 ± 2 °C), with a 12 h light-dark cycle period and access to pelleted food and water *ad libitum.*

1. Preparation of the material and instruments

1.1. Connect the heat blanket to maintain the temperature of the operation area and the mouse body temperature during anesthesia at 37 °C.

1.2. Autoclave scissors and forceps, prepare 70% ethanol solution and dexpanthenol eye ointment, and keep several pieces of cotton and 5-0 coated braided polyester suture ready for use. Prepare a 1 mL syringe with 0.9% saline solution (without needle) to keep the animal's incision site hydrated. Prepare the anesthesia gas (100% O_2 + isoflurane).

1.3. Prepare a holder for the laser Doppler probe by cutting the tip of a 10 µL pipet tip (3–5 mm length)

2. Preparation of the laser Doppler

2.1. Inject analgesia to the mouse 30 min before the surgery (4 mg/kg Carprofen und 0,1 mg/kg Buprenorphine, intraperitoneally).

2.2. Anesthetize the mouse by placing it in the induction chamber with an isoflurane flow rate of 4% until the cessation of spontaneous body movement and vibrissae.

2.3. Place the mouse in a prone position in the operation area with its nose in the anesthesia mask. Maintain isoflurane concentration at 4% for another minute, then reduce it and keep it at 2%.

2.4. Set the associated feedback-controlled heating pad for maintaining the mouse body temperature at 37 °C, and gently insert the rectal probe to monitor the temperature throughout the surgical procedures..

2.5. Apply dexpanthenol eye ointment on both eyes.

2.6. Disinfect the skin and hair surrounding the left eye and ear with a disinfectant.

2.7. Cut the scalp between the left ear and the eye (1 cm long) to expose the skull bone.

2.8. Cut and retire the temporal muscle to visualize the MCA beneath the skull.

2.9. Fix the outer part of the tip holding the laser Doppler probe/fiber on top of the left MCA with glue and close the skin so that the skin is glued as well. Apply 2–3 drops of hardener glue to speed up the process. Make sure that the laser Doppler fiber is not glued and can be easily removed from the tip holder at any time.

3. Transient MCAo model (occlusion)

3.1 Turn the mouse into the supine position. Put the snout into the anesthesia cone and fix the paws with tape.

3.2 Disinfect the skin and hair surrounding the chest and make a 2-cm-long midline incision in the neck.

3.3 Use forceps to pull the skin, submandibular gland, and sternomastoid muscle apart. Use retractors to expose the surgical field and find the left common carotid artery (CCA). Dissect the CCA free from connective tissue and surrounding nerves (without harming the vagal nerve) and perform a transient ligation before the bifurcation.

3.4 Dissect the external carotid artery (ECA) and tie a permanent knot at the most distal visible part. Place another suture under the ECA, close to the bifurcation, and prepare a loose knot to be used later.

3.5 Dissect the internal carotid artery (ICA) and place a microvascular clip on it, 5 mm over the bifurcation. Make sure not to damage the vagal nerve.

3.6 Cut a small hole into the ECA between the tight and the loose ligations; be careful not to cut the entire ECA.

3.7 Introduce the filament and advance it towards the CCA. Tighten the loose ligation in the ECA around the lumen to momentarily secure the filament in that position and avoid bleeding when removing the microvascular clip.

3.8 Remove the microvascular clip and insert the filament through the ICA until the origin of the MCA is reached by detecting a sharp reduction (>80%) in the cerebral blood flow as measured by the laser Doppler. Fix the filament in this position by further tightening the knot around the ECA.

NOTE: When the filament goes toward the appropriate direction, it advances smoothly, and no resistance should be observed.

3.9 Record laser Doppler values before and after filament insertion.

3.10 Remove the retractor and relocate the sternomastoid muscle and the submandibular gland before suturing the wound. Remove the laser Doppler probe, and place the animal in a recovery chamber at 37 °C for 1 h (until filament removal).

4. Transient MCAo model (Reperfusion)

4.1. Anesthetize the mouse by placing it in the induction chamber with an isoflurane flow rate of 4% until the cessation of spontaneous body movement and vibrissae.

4.2. Apply dexpanthenol eye ointment on both eyes.

4.3. Place the mouse in a prone position in the operation area with its snout in the anesthesia mask. Maintain isoflurane concentration at 4% for another minute, then reduce it and keep it at 2%. Fix the animal´s paws with tape.

4.4. Insert the laser Doppler probe into the probe holder.

4.5. Remove the wound suture, use forceps to pull the skin, the submandibular gland, and the sternomastoid muscle apart. Use retractors to expose the surgical field.

4.6. Loosen the ECA suture that tightens the filament, and gently pull the filament. Avoid damaging the silicone-rubber coating of the filament during the removal.

4.7. Tightly tie the ECA suture.

4.8. Confirm the increase in the cerebral blood flow in the laser Doppler device (>80% of the initial value before reperfusion).

4.9. Record laser Doppler values before and after filament removal.

4.10. Open the transient ligation before the bifurcation from the CCA.

4.11. Remove the retractor, and relocate the sternomastoid muscle and the submandibular gland before suturing the wound. Place the animal in a recovery chamber at 37 °C for 1 h to recover from anesthesia.

4.12. After recovery, return the mice to their cages in a temperature-controlled room.

4.13. Take care of the animals by adding wet food pellets and hydrogel in small Petri dishes on the cage floor until day 3 after the surgery.

4.14. Inject analgesia every 12 h for 3 d after surgery (4 mg/kg Carprofen and 0.1 mg/kg Buprenorphine).

5. Sham operation

5.1. Perform all procedures as described above, including the ligation of the arteries and the introduction of the filament (steps 1–3.7).

5.2. Remove the filament immediately after its insertion. Then, place the animal in the recovery chamber for 1 h.

5.3. Place the animal in the operation area again, and remove the transient ligation of the CCA to ensure complete cerebral blood flow restoration.

5.4. Suture the wound, and place the animal in a recovery chamber at 37 °C for 1 h to recover from anesthesia. After recovery, return the mice to their cages in a temperature-controlled room.

5.5. Take care of the animals by adding wet food pellets and hydrogel in small Petri dishes on the cage floor until day 3 after surgery.

5.6. Inject analgesia every 12 h for 3 d after surgery (4 mg/kg Carprofen and 0.1 mg/kg Buprenorphine).

6. Neuroscore

6.1. Perform the Neuroscore always at the same time of the day, and use surgical clothes to maintain a “neutral smell” between individual surgeons.

6.2. Let the mice rest for 30 min in the room with an “open” cage before the test.

6.3. Observe each item in **Table 1** and **Table 2** for 30 s.

7. Intracardiac perfusion

7.1. Prepare a 20 mL syringe containing phosphate-buffered saline (PBS)-heparin (2 U/mL) and place it 1 m above the bench to facilitate gravity-driven perfusion. (OPTIONAL: Perform intracardiac perfusion with 4% paraformaldehyde (PFA) using a 20 mL syringe containing 4% PFA in PBS, pH 7.4).

7.2. Inject 100 µL of ketamine and xylazine (120/16 mg/kg body weight, respectively). Wait 5 min and confirm the cessation of spontaneous body movement and vibrissae.

7.3. Fix the animal in a supine position, and disinfect the abdominal body surface with 100% ethanol.

7.4. Make a 3-cm-long incision into the abdomen; cut the diaphragm, the ribs, and sternum to visualize the heart completely.

7.5. Make a small incision in the right atrium, and insert the perfusion cannula into the left ventricle.

7.6. Perfuse with 20 mL of PBS-heparin.

7.7. After perfusion, decapitate the animal and remove the brain.

7.8. Freeze the brain on powdered dry ice and store at -80 °C until further use.

8. Infarct volumetry

8.1. For cryosectioning, use a cryostat to cut the brains into 20-µm-thick sections every 400 µm. Place the sections on slides, and store the slides at −80 °C until use.

8.2. Cresyl violet (CV) staining

8.2.1. Prepare the staining solution by stirring and heating (60 °C) 0.5 g of CV acetate in 500 mL of H_2O until the crystals are dissolved. After the solution has cooled, store it in a dark bottle. Reheat to 60 °C and filter before every use.

8.2.2. Let the slides dry at room temperature for 30 min. Immerse them in 95% ethanol for 15 min, in 70% ethanol for 1 min, and then in 50% ethanol for 1 min.

8.2.3. Immerse the slides in distilled water for 2 min; refresh the distilled water and place the slides in the water for 1 min. Afterward, immerse the slides in the pre-heated staining solution for 10 min at 60 °C. Wash the slides twice in distilled water for 1 min.

8.2.4. Immerse the slides in 95% ethanol for 2 min. Place them in 100% ethanol for 5 min; refresh the 100% ethanol and place the slides again in the ethanol for 2 min. Afterward, cover the slides with a mounting medium.

8.2.5. Analysis (**Figure 4C**)

8.2.5.1. Scan the slides and analyze the indirect infarct volume by the Swanson method[12] to correct for edema by using equation (**1**).

(Ischemic area) = (ischemic region)-((ipsilateral hemisphere)-(contralateral hemisphere)) (**1**)

REPRESENTATIVE RESULTS:

The model described here is a modification of the commonly used "filament" stroke model, which consists of introducing a silicon-coated filament through the ECA to block the origin of the MCA transiently. After removing the filament, only the ECA is permanently occluded, allowing complete blood restoration in the CCA and ICA (**Figure 1**). Moreover, this paper describes a method for measuring the cerebral blood flow during both occlusion and reperfusion procedures by fixing a cannula connected to the laser Doppler probe at the skull over the MCA territory.

Because the blood flow in the CCA is restored after removing the filament, complete reperfusion of the brain occurs (**Figure 2**), similar to the situation observed after successful mechanical thrombectomy in human patients. The mortality rate during the surgery is <5% when performed by trained surgeons. At these early time points, animals generally present severe postural and movement deficits, general weakness, and loss in body weight[13]. These severe deficits are transient, and the animals show improved activity after approximately 1 week; thus, the deficits are more specific for focal neurological symptoms.

Behavioral deficits after MCA occlusion were assessed by the composite Neuroscore[14]; general and focal deficits were measured 24 h and 3 d after surgery. The general Neuroscore has 5 items (**Table 1**), including the evaluation of the fur, ears, eyes, posture, and spontaneous activity, with a maximum score of 18. The focal Neuroscore comprises 7 items (**Table 2**), including the evaluation of body symmetry, gait, climbing, circling behavior, forelimb symmetry, compulsory cycling, and whiskers response, with a maximum score of 28. This composite scale ranges from 0 (no deficits) to 46 (severe impairments). Stroke animals presented a significant change in the composite and focal Neuroscore, but not in the general Neuroscore when compared to sham animals (**Figure 3**).

Infarct volumetry was also performed using cresyl violet staining of coronal serial brain sections 24 h after stroke induction. The infarct volume mean was 61.69 mm^3, representing 48% of the affected brain hemisphere (**Figure 4**). When performed by a trained surgeon, the variability of this stroke model is low, with a coefficient of variation of 6%. The lesion area includes the somatosensory and motor cortex as well as subcortical structures such as the striatum (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Scheme for the access and intraluminal MCA occlusion. The filament (dotted line) is inserted between the proximal and distal suture knots in the ECA and advanced along the ICA until it reaches the origin of the MCA (see inset). Once in place, the ECA is ligated with a suture to fix the filament. Abbreviations: ACA = anterior cerebral artery; BA = basilar artery; CCA = common carotid artery; ECA = external carotid artery; ICA = internal carotid artery; MCA = middle cerebral artery; PCA = posterior communicating artery; PTG = pterygopalatine artery. This figure has been modified from Jackman et al.[15].

Figure 2: Blood flow during occlusion and reperfusion. Blood flow is registered before and after filament insertion and before and after filament removal. A reduction in the blood flow was observed during the occlusion and the restoration of the blood flow during the reperfusion. Every color represents one animal. Abbreviations: MCA = middle cerebral artery; CBF = cerebral blood flow; A.U. = arbitrary units.

Figure 3: Neuroscore for functional deficits after tMCAo. (**A**) Total, (**B**) focal, and (**C**) general Neuroscore before and 24 h and 3 d after tMCAo. Open bars: sham; Black bars: tMCAo. n=10 per group. *$p < 0.05$. Abbreviations: tMCAo = transient middle cerebral artery occlusion; BL = before tMCAo.

Figure 4: Volumetric infarct analysis and infarct outcome 24 h after tMCAo. (**A**) Representative cresyl violet-stained coronal brain sections every 400 µm at 24 h after tMCAo. Dashed lines demarcate the lesion area. (**B**) Analysis of infarct volume of 10 brains (each dot representing one individual brain) 24 h after tMCAo. The horizontal red line represents the mean (61.69 mm^3), error bars indicate standard deviation (3.78 mm^3). (**C**) Representative image for infarct volume calculation from a cresyl violet coronal section. Blue = Contralateral hemisphere; Red = Ipsilateral hemisphere; Pale striped area = Ischemic region.

Table 1: General Neuroscore. Animals received between 0 and 4 points, depending on the severity, for each of the five general deficits measured. The scores on the different areas are then added to provide a total general score ranging from 0 to 18. This table has been modified from Clark et al.[14] . Abbreviation: OBT = open benchtop.

Table 2: Focal Neuroscore. Animals received between 0 and 4 points depending on the severity for each of the seven general deficits measured. The scores on the different areas are then added to provide a total focal score ranging from 0 to 28. This table has been modified from Clark et al.[14] . Abbreviation: OBT = open benchtop.

DISCUSSION:

The present protocol describes an experimental stroke model based on the consensus agreement of a German multicenter research consortium ("ImmunoStroke") to establish a standardized transient MCAo model. The transient MCAo model established by introducing a silicon-coated filament through the ECA until the origin of the MCA is one of the most commonly used stroke models to achieve arterial reperfusion after a delimitated occlusion period. Thus, this procedure

can be regarded as a translationally relevant stroke model.

The “filament model” presented in the video has some advantages compared with other previously described stroke models, such as not needing craniotomy and achieving complete reperfusion of the occluded vessel. However, the complexity of the surgery could be considered as a limitation as it includes invasive surgery and a precise manipulation of the different arteries very close to the trachea and the vagal nerve. Moreover, the long exposure of the animal to anesthetics might be a critical factor to consider, as the impact of anesthetics on neuroprotection and stroke outcome has already been well documented[16]. Although this complex surgical procedure cannot be completed as quickly as other brain ischemia models, it can be completed in ~20 min when performed by a trained surgeon.

In contrast to previously described “filament” stroke protocols[17], the method described here also allows the measurement of the cerebral blood flow during both occlusion and reperfusion phases. Monitoring the blood flow during reperfusion might be an important parameter for preventing stroke reperfusion injury[18], which is known to cause deleterious consequences in patients who underwent pharmacologic or endovascular interventions for recanalization of the thrombosed vessels. Despite the existing discrepancies between the consequences of cerebral blood flow restoration after MCAo[19], the variability of blood flow restoration after stroke can influence the pathophysiological and biochemical events in the brain as well as the infarct volume and the neurological deficits of stroke mice[20]. Therefore, in this model, complete blood flow restoration and its recording are requirements to guarantee reproducible infarcts among mice, especially in translational stroke studies.

The overall mortality during surgery is <5% and is mainly caused by anesthesiology complications, bleeding, or sacrifice due to pre-defined exclusion criteria. In contrast, this stroke model presents a moderate mortality rate within the first 24–48 h after stroke induction, which might increase the number of animals needed per experiment to achieve a proper cohort of stroke mice. In terms of infarct volume, this model induces large infarcts, with ~50% of the hemisphere affected by the ischemia. It also produces brain swelling, affecting different regions of the brain, including the cortical and subcortical regions.

To achieve low variability and high reproducibility of this stroke model, we suggest that the following exclusion criteria be taken into account: 1) operation time > 20 min; 2) >20% of blood flow reduction when CCA ligated (step 3.3); 3) reduction in blood flow during occlusion <80% of the initial pre-occlusion value; and 4) increase in blood flow 10 min after reperfusion rate <80% compared to the pre-reperfusion value. For an experienced and trained surgeon, no animals are excluded due to the operation time criterion. However, 10–15% of the animals show a 20% reduction in the blood flow when the CCA is ligated, and 5–10% do not have an adequate reduction or increase in blood flow during occlusion or reperfusion, respectively. Therefore, the success rate after excluding animals according to these criteria is approximately 75–85%.

In addition, animals are examined daily after MCAo (body weight, temperature, and basic physiological behavior) to control for sickness behavior, pain, or discomfort. In addition to this

general care, several tests for specific behavioral analysis after focal brain ischemia have been developed despite all the different tests to evaluate sensorimotor dysfunction, such as the Rotarod test[21], Sticky label test[22], Corner test[23], or the Cylinder test[24]. Here, animals selected for the establishment of this stroke model were evaluated for focal and general deficits because stroke also induces cytokine-sickness behavior independent of focal (sensory or motor) deficits[25]. Taken together, the "filament" stroke model described here is a valuable model for basic and translational stroke research. This model is proposed as a standardized stroke model to be used to harmonize stroke models across laboratories.

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DISCLOSURES:

The authors have no competing interests to disclose.

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Figure 1

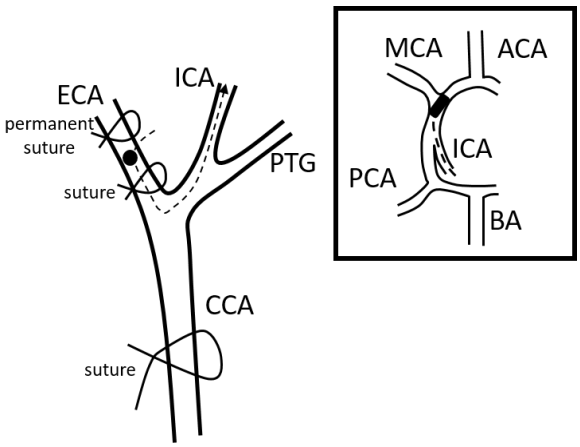
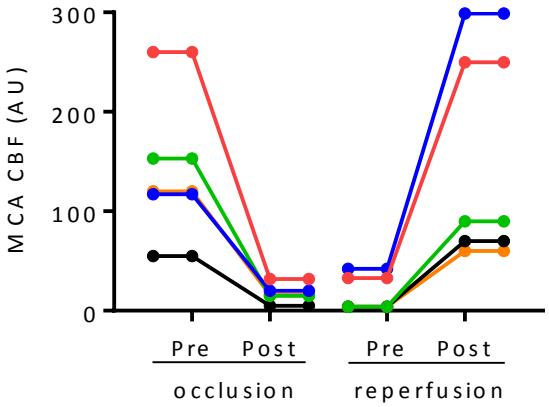


Figure 2


300
200
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Pre
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reperfusion

Figure 3

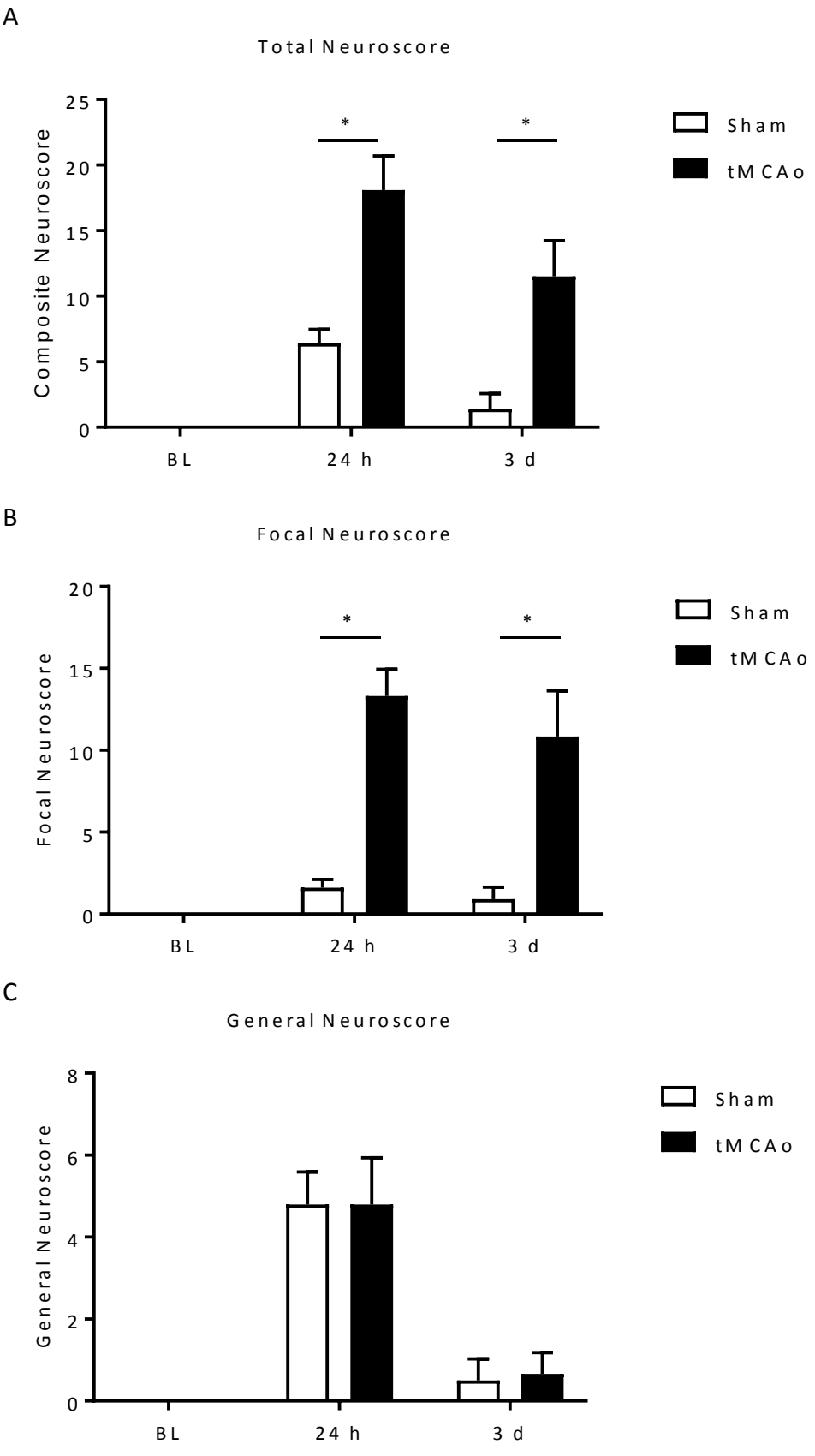
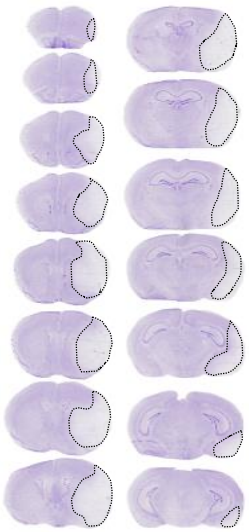
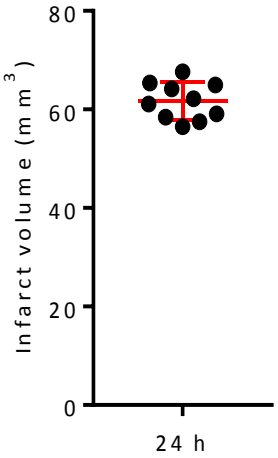

A
Total Neuroscore
Composite Neuroscore
25
20
15
10
5
0
*
*
BL
24 h
3 d
Sham
tMCAo
B
Focal Neuroscore
Focal Neuroscore
20
15
10
5
0
*
*
BL
24 h
3 d
Sham
tMCAo
C
General Neuroscore
General Neuroscore
8
6
4
2
0
BL
24 h
3 d
Sham
tMCAo

Figure 4

A



B



C

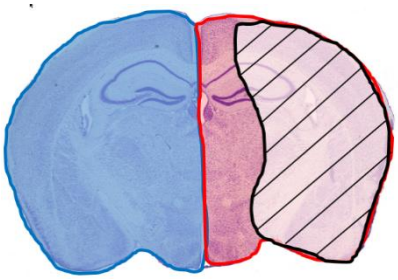


Table 1

General Neuroscore	**Spontaneos activity** (mouse on OBT)	
	Posture (place the mouse on the palm and swing gently)	
	Eyes (mouse on OBT)	
	Ears (mouse on an open bench top)	
	Hair	
	Total score for gen	

	(normal=0 max=18

Time-point of scoring
0. Hair neat and clean
1. Localized piloerection and dirty hair in 2 body parts (nose and eyes)
2. Piloerection and dirty hair in >2 body parts
0. Normal (ears are stretched laterally and behind, they react by straightening up following noise)
1. Stretched laterally but not behind (one or both), they react to noise
2. Same as 1. NO Reaction to noise.
0. Open, clean and quickly follow the surrounding environment
1. Open and characterized by aqueous mucus. Slowly follow the surrounding environment
2. Open and characterized by dark mucus
3. Ellipsoidal shaped and characterized by dark mucus
4. Closed
0. The mouse stands in the upright position with the back parallel to the palm. During swing, it stands rapidly.
1. The mouse stands humpbacked. During the swing, it flattens the body to gain stability.
2. The head or part of the trunk lies on the palm.
3. The mouse lies on one side, barely able to recover the upright position.
4. The mouse lies in a prone position, not able to recover the upright position.
0.The mouse is alert and explores actively.
1.The mouse seems alert, but it is calm and sluggish.
2.The mouse explores intermittently and sluggishly.
3.The mouse is somnolent and numb, few movements on-the-spot.
4.No spontaneous movements
eral scoring

)

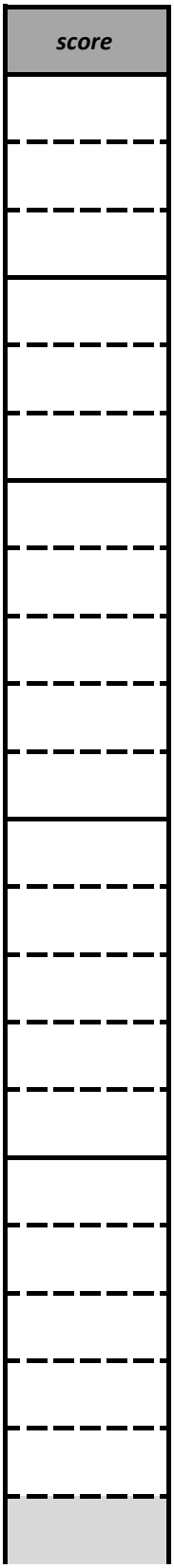

score

Table2

Focal Neuroscore	**Body symmetry** (mouse on OBT, observe the nose-tail line)	
	Gait (mouse on OBT. Observed undisturbed)	
	Climbing (mouse on a 45° surface. Place the mouse in the center of the gripping surface)	
	Circling behavior (mouse on OBT, free observation)	
	ymmetry (mouse nded by tail)	

Total score for focal def (normal=0 max=28)	**Whisker response** (mouse on the OBT)	**Compulsory circling** (forelimbs on bench, hindlimbs suspended by the tail: it reveals the presence of the contralateral limb palsy)	**Forelimb s** suspe

Time-point of scoring
0. Normal (Body: normal posture, trunk elevated from the bench, with fore and hindlimbs leaning beneath the body. Tail: straight)
1. Slight asymmetry (Body: leans on one side with fore and hindlimbs leaning beneath the body. Tail: slightly bent)
2. Moderate asymmetry (Body: leans on one side with fore and hindlimbs stretched out. Tail: slightly bent)
3. Prominent asymmetry (Body: bent, on one side lies on the OBT. Tail: bent)
4. Extreme asymmetry (Body: highly bent, on one side constantly lies on the OBT. Tail: highly bent)
0. Normal (gait is flexible, symmetric and quick)
1. Stiff, inflexible (humpbacked walk, slower than normal mouse)
2. Limping, with asymmetric movements
3. Trembling, drifting, falling
4. Does not walk spontaneously (when stimulated by gently pushing the mouse walks no longer than 3 steps)
0. Normal (mouse climbs quickly)
1. Climbs with strain, limb weakness present
2. Holds onto slope, does not slip or climb
3. Slides down slope, unsuccessful effort to prevent fail
4. Slides immediately, no effort to prevent fail
0. Absent circling behavior
1. Predominantly one-side turns
2. Circles to one side, although not constantly
3. Circles constantly to one side
4. Pivoting, swaying, or no movement
0. Normal
1. Light asymmetry: mild flexion of contralateral forelimb
2. Marked asymmetry: marked flexion of contralateral limb, the body slightly bends on the ipsilateral side

3. Prominent asymmetry: contralateral forelimb adheres to the trunk
4. Slight asymmetry, no body/limb movement
0. Absent. Normal extension of both forelimbs
1. Tendency to turn to one side (the mouse extends both forelimbs, but starts to turn preferably to one side)
2. Circles to one side (the mouse turns towards one side with a slower movement compared to healthy mice)
3. Pivots to one side sluggishly (the mouse turns towards one side failing to perform a complete circle)
4. Does not advance (the front part of the trunk lies on the bench, slow and brief movements)
0. Normal
1. Light asymmetry (the mouse withdraws slowly when stimulated on the contralateral side)
2. Prominent asymmetry (no response when stimulated to the contralateral side)
3. Absent response contralaterally, slow response when stimulated ipsilaterally
4. Absent response bilaterally
icits

score

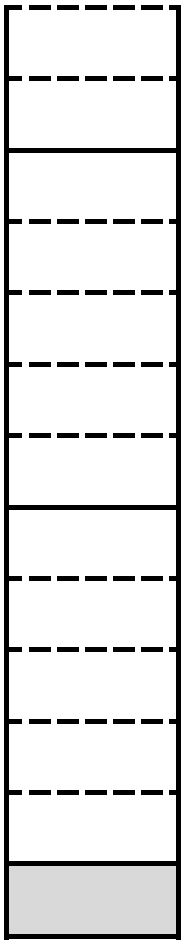


Table of Materials

Click here to access/download;Table of Materials;JOVE_tMCAo_GL_Materials.xls

Name of Material/ Equipment	Company	Catalog Number
45º ramp	H&S Kunststofftechnik	
5/0 threat	Pearsalls	10C103000
5 mL Syringe	Braun	
Acetic Acid	Sigma Life Science	695092
Anesthesia system for isoflurane	Drager	
Bepanthen pomade	Bayer	
C57Bl/6J mice	Charles River	000664
Clamp	FST	12500-12
Clip	FST	18055-04
Clip holder	FST	18057-14
Cotons	NOBA Verbondmitel Danz	974116
Cresyl violet	Sigma Life Science	C5042-10G
Cryostat	Thermo Scientific CryoStarNX70	
Ethanol 70%	CLN Chemikalien Laborbedorf	521005
Ethanol 96%	CLN Chemikalien Laborbedorf	522078
Ethanol 99%	CLN Chemikalien Laborbedorf	ETO-5000-99-1
Filaments	Doccol	602112PK5Re
Fine 45 angled forceps	FST	11251-35
Fine forceps	FST	11252-23
Fine Scissors	FST	14094-11
Glue	Orechseln	BSI-112
Hardener Glue	Drechseln & Mehr	BSI-151
Heating blanket	FHC DC Temperature Controller	
Isoflurane	Abbot	B506
Isopentane	Fluka	59070
Ketamine	Inresa Arzneimittel GmbH	
Laser Doppler	Perimed	PF 5010 LDPM, Peri
Laser Doppler probe	Perimed	91-00123
Phosphate Buffered Saline pH: 7.4	Apotheke Innestadt Uni Munchen	P32799
Recovery chamber	Mediheat	
Roti-Histokit mounting medium	Roth	6638.1
Saline solution	Braun	131321

Scalpel	Feather	02.001.30.011
Silicon-coated filaments	Doccol	602112PK5Re
Stereomicropscope	Leica	M80
Superfrost Plus Slides	Thermo Scientific	J1800AMNZ
Vannas Spring Scissors	FST	15000-00
Xylacine	Albrecht	

Comments/Description
height: 18 cm

iflux System 5000

Modeling stroke in mice: transient middle cerebral artery occlusion via the external carotid artery

We would like to thank the reviewers and editors for the very positive general evaluation of our manuscript and the constructive comments. Please find below a point-by-point reply to all individual comments:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: As suggested, we reviewed the manuscript thoroughly and confirmed that there are no spelling or grammatical problems.

2. Please revise the following lines to avoid previously published work: 30-31, 38-39, 50-52, 56-57, 59-64, 108-110, 123-129, 142-144, 208-210, 292-315, 345-347, 354-356, 358-360, 362-367, 380-382, 424-425.
Response: As suggested, we have reviewed the different selected sections and added the appropriate references. We would like to point out that no references were added in the summary and abstract.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Response: As suggested, we substituted all personal pronouns in the new version of the manuscript.

4. Line 127-129: Please specify the size and type of the suture material. What volume of syringe is used? How much volume of saline is taken in the syringe?
Response: As suggested, we have now specified the size and type of suture material (5-0 coated braided polyester suture line: 124). We also added information on the type of syringe used (1 mL syringe, line: 125). However, regarding the volume of saline taken in the syringe, we would like to point out that saline solution is used throughout the surgical procedure to maintain the operation area hydrated and there is no limitation on the specific volume used for each animal (line: 125-126).

5. Line 136-137: Please specify the route of injection.
Response: As suggested, we have now added the injection method (intraperitoneally) in the protocol (line: 134).

6. Line 159: Please mention how the skin is closed?
Response: As suggested, we have now better clarified how the skin is closed. In brief, at the same time that we glue the tip holder to the skull, the skin is also put in place and glued accordingly (line: 156).

7. Line 243-247/252-255: Please ensure that the Protocol section consists of numbered steps. We cannot have non-numbered paragraphs/steps/headings/subheadings. Add “NOTES” if necessary.
Response: As suggested, we modified the protocol so that all the steps are now numbered (line: 243-262/266-273).

8. For SI units, please use standard abbreviations when the unit is preceded by a numeral

throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2.
Response: As suggested, we have changed all the abbreviations according to the international system of units (SI).

9. Please do not highlight anesthesia and euthanasia steps in the protocol.
Response: As suggested, we have removed the anesthesia and euthanasia steps from the highlighted steps.

10. Please include some limitations of the protocol in the discussion.
Response: As suggested, we added some limitations of the protocol in the discussion. In brief, "the complexity of the surgery could be considered as a limitation, since it includes an invasive surgery and a precise manipulation of the different arteries very close to the trachea and the vagal nerve. Also, the long exposure of the animal to anesthetics might be a critical factor to also take into account, as the impact of anesthetics on neuroprotection and stroke outcome has already been well documented." (line: 410-414)

11. Please do not use the &-sign or the word "and" when listing authors in the references. Please title case and italicize journal titles and book titles. Do not use any abbreviations.
Response: For the reference list, we have used the ENDNOTE's JOVE citation style, with no additional modifications, as written in the guidelines.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Response: Although Figure 1 is our own production and it is a simple schematic representation of the vessels involved in the surgery and how we introduce the Doccol® filament to occlude the MCA, we adapted from a previous publication (Jackman *et al.*, 2011) and we now cited in the figure legend (line: 379).

13. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.
Response: As suggested, we have now removed the titles from the uploaded figures.

14. Please sort the Table of Materials in alphabetical order.
Response: As suggested, we now sorted the Table of Materials alphabetically by the name of the material.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript mainly focuses on the establishment of a stroke model by the method of transient middle cerebral artery occlusion via the external carotid artery. The research method summarizes some commonly used methods and conducts a comprehensive and accurate

summary. The detailed steps are convenient for researchers to understand and learn MCAO, which has extremely high reference value. In order to make it easier for researchers to understand, the manuscript needs some improvements.

Major Concerns:

none

Minor Concerns:

1. Line 171, change the sentence" Use retractors to find the left common carotid artery (CCA) and expose the surgical field" to the "Use retractors to expose the surgical field and find the left common carotid artery (CCA)".
Response: As suggested by the Reviewer, we have changed the original sentence in line 171 for the suggested one (line: 170).

2. Line 315, it is recommended to add a picture to show the formula more intuitively.
Response: We thank the Reviewer for this recommendation. We have added a schematic representation to better show how the infarct volume is calculated (Figure 4C) (line: 323).

3. MCAO or MCAo, needs to be coherent.
Response: As suggested, we reviewed the manuscript thoroughly to make abbreviations consistent.

Reviewer #2:

Manuscript Summary:

The manuscript proposes a standardized transient MCAO mouse model based in introducing a silicon-coated filament in the external carotid artery, which is pushed until de Circle of Willis through the internal carotid artery to occlude the middle cerebral artery (MCA). This occlusion is monitored with a laser Doppler which allows detecting the blood flow through MCA. A modified neurological scale previously published is described to measure deficit analysis and procedures for measuring infarct volumetry based on Nissl staining are also described.

Major Concerns:

Line 86. There is a report Liu et al., 2012, PMID: 23272656 (doi: 10.1186/1471-2202-13-154) that compared two different procedures of performing MCAO one of the similar to that described in this manuscript and another method that pushes the filament from the common carotid artery which is much easier to perform. These authors indicate similar results in both methods. I am aware that the study was performed in rat, but I think it is worth referencing this alternative method that is easier to perform both in rat and mice.
Response: As suggested, we have now mentioned this issue in the discussion section. “Despite existing discrepancies between the consequences of cerebral blood flow restoration after MCAo[19], it has been described that the variability of blood flow restoration after stroke can influence the pathophysiological and biochemical events in brain, as well as the infarct volume and the neurological deficits of stroke mice[20]” (line:423-427).

More details should be indicated in some of the steps. Thus in step 1.2, material should be autoclaved, and ethanol 70% should also be prepared.
Response: As suggested by the Reviewer, we have now added more detailed information in step 1.2 (line: 123-126)

Step 2.9. It should be indicated if the procedure should be performed in the left or right side to be consistent with the artery to be occluded, the neuroscore, and the area of the brain damaged.
Response: As suggested, we have now specified that the laser Doppler probe is placed on the left side of the animal, to be consistent with the side of MCAo and properly record the drop in the cerebral blood flow (line: 155).

Step 3.2 The neck area where the incision will be made should also be shaved and disinfected
Response: Although we agree with the Reviewer, we cannot add the following statement in the manuscript. The protocol we are describing in this manuscript represents the consensus across different laboratories conforming the "ImmunoStroke" research consortium, all who have agreed on these procedures, including the fact of not shaving the mice before starting the surgery, but meticulously disinfect the area surrounding the chest. In addition, the protocol in its current form is also in line with good laboratory practices in all participating institutions of the aforementioned research consortium.

Step 3.3. More details explaining the skin, salivary glands and muscle handling should be done. The dissection of the vagal nerve should be more careful (this part should be explained carefully in the video)
Response: According to the Reviewer's suggestion, we have now better clarified how to handle the sternomastoid muscle and the submandibular gland to expose the surgical field (step 3.3, line: 169). Moreover, we will emphasize how to carefully dissect the vagal nerve in the video.

Step 3.6 This step is critical and requires a more careful explanation of the procedure of making the hole in the ECA.
Response: We agree with the Reviewer that this is a critical step. However, we will emphasize how to carefully make the hole in the ECA in the video, which will be most helpful for the audience.

Step 3.7. The filament advance towards the Willis circle is not always smooth. Some suggestions to make easier the filament through the artery could help the readers. The second sentence in step 3.7 is confusing since the filament is still being pushed toward the Wills circle.
Response: We completely agree with the Reviewer. We are fully aware that if the filament introduction is not smooth, it very likely means that the filament goes into the pterygopalatine artery and not to the internal carotid artery (ICA). In such case, the surgeon needs to redirect the filament and properly introduce it into the ICA. However, if the filament goes to the appropriate direction and moves through the ICA, the filament always advance smoothly and no resistance is found (Step 3.8 line: 188-192).
Regarding the second sentence in step 3.7, we have now tried to better clarify in that sentence that the filament is secured in place to avoid bleeding when removing the microvascular clip in the next step. The filament is later flipped towards to the direction of the ICA until it reaches the MCA, where it is properly fixed (step 3.8).

Step 3.11. The re-position of the different tissues, muscles, glands and skin should be indicated. An alternative to the sutures is the use of surgical staples before the reperfusion and the suture could be performed after removing the filament.

Response: As suggested, we have clarified how sternomastoid muscle and the submandibular gland is done before suturing the skin. Regarding the use of surgical staples, however, all researchers conforming the "ImmunoStroke" research consortium agreed that suturing the animals is in our case better that the surgical staples, since those placed on the chest may be uncomfortable for the animal.

Step 4.5. Again the handling of skin, glands and muscle should be indicated or at least referred to a previous step.
Response: As suggested, we have clarified again how sternomastoid muscle and the submandibular gland handling is done (step 4.5, line: 215)

Step 4.6. Removing the filament should be done very carefully since an improper removal can prevent reperfusion (in our experience in rats).
Response: We completely agree with the Reviewer that filament removal has to be done meticulously to avoid the rupture of the filament. However, we would like to point out that in our hands, and on behalf of all researchers conforming the "ImmunoStroke" research consortium, inappropriate reperfusion is observed in very few cases (~5%) and it is not associated with the way in which the filament is removed.

Step 4.11. Again the re-position of the tissues should be indicated. Also, disinfection after the suture is recommended.
Response: As suggested, we have now clarified how sternomastoid muscle and the submandibular gland are handled and positioned back into place (step 4.11, line: 230-232)

Section 7 Consider renaming "Perfusion" to "Intra-aortic perfusion" or similar to avoid confusion with the term reperfusion used before
Response: We agree with the Reviewer that both terms could lead to confusion. Accordingly, we renamed "Perfusion" to "Intracardiac perfusion".(line: 276)

In section 7 Consider the possibility of perfusion with 4% paraformaldehyde for better fixing of the brain (proper cautions should be indicated in this case)
Response: As suggested, we added a note stating the possibility to perfuse the animal with 4% PFA, according to the specific experimental aim (line: 279-280).

Reviewer #3:

Manuscript Summary:

The manuscript proposed a standardized transient MCA occlusion mouse model in which completely recover blood flow when removing the filament.

Major Concerns:

This manuscript is well written and helpful for scientists to reproduce the focal cerebral ischemia in mice. However, the similar MCA occlusion mouse model has already been published in JoVE (Rousselet E, Kriz J, Seidah NG. Mouse model of intraluminal MCAO: cerebral infarct evaluation by cresyl violet staining. J Vis Exp. 2012 Nov 6;(69):4038). This previous paper should be sited and discussed in the manuscript.
Response: As suggested, we have now discussed and cited the paper the Reviewer mentions (line: 418-429). "In contrast with previously described "filament" stroke protocols[17], the

method here described also allows the measurement of the cerebral blood flow during both, occlusion and reperfusion phases. Monitoring the blood flow during reperfusion might be an important parameter for preventing stroke reperfusion injury[18], which in clinics is known to cause deleterious consequences in patients that underwent pharmacologic or endovascular interventions for recanalization of the thrombosed vessels. Despite existing discrepancies between the consequences of cerebral blood flow restoration after MCAo[19], it has been described that the variability of blood flow restoration after stroke can influence the pathophysiological and biochemical events in brain, as well as the infarct volume and the neurological deficits of stroke mice[20]. Therefore, in the model here described, a complete blood flow restoration and its recording are requirements to guarantee reproducible infarcts among mice, especially when aiming at conducting translational stroke studies."

Minor Concerns:

Authors described that "the overall mortality during surgery is less than 5% (line 409)", but suggested exclusion criteria "1)Operation time longer than 20 min; 2)More than 20% of blood flow reduction of the initial value when CCA occluded; 3)Reduction of blood flow during occlusion below 80% of the initial pre-occlusion value, and 4) Reperfusion rate after 10 min below 80% of the pre-reperfusion value.(lines 546-551)". Please describe success rate after excluding these cases.

Response: As suggested, we have now clarified the success rate after rejecting the surgeries according to the exclusion criteria. "For an experienced and trained surgeon, no animals are excluded due to the operation time. However 10-15% of the animals show a 20% reduction of the blood flow when the CCA is ligated and 5-10% do not have an adequate reduction or increase of the blood flow during occlusion or reperfusion, respectively. Therefore, the success rate after excluding animals according to these criteria is approximately 75-85%". (lines 445-449).