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## An easy and efficient high output method to isolate cerebral pericytes from mouse

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**TITLE:**

# A High Output Method to Isolate Cerebral Pericytes from a Mouse

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**KEYWORDS:**

Primary cerebral pericytes, isolation, mouse, dextran

**SUMMARY:**

We present a protocol for the extraction of murine cerebral pericytes. Based on an antibiotic-free enrichment oriented pericyte extraction, this protocol is a valuable tool for in vitro studies providing high purity and high yield, thus decreasing the number of experimental animals used.

**ABSTRACT:**

In recent years, cerebral pericytes have become the focus of extensive research in vascular biology and pathology. The importance of pericytes in blood brain barrier formation and physiology is now demonstrated but its molecular basis remains largely unknown. As the pathophysiological role of cerebral pericytes in neurological disorders is intriguing and of great importance, the in vitro models are not only sufficiently appropriate but also able to incorporate different techniques for these studies. Several methods have been proposed as in vitro models for the extraction of cerebral pericytes, although an antibiotic-free protocol with high output is desirable. Most importantly, a method that has increased output per extraction reduces the usage of more animals.

Here, we propose a simple and efficient method for extracting cerebral pericytes with sufficiently high output. The mouse brain tissue homogenate is mixed with a BSA-dextran solution for the separation of the tissue debris and microvascular pellet. We propose a three-step separation followed by filtration to obtain a microvessel rich filtrate. With this method, the quantity of

microvascular fragments obtained from 10 mice is sufficient to seed 9 wells (9.6 $cm^2$ each) of a 6-well plate. Most interestingly with this protocol, the user can obtain 27 pericyte rich wells (9.6 $cm^2$ each) in passage 2. The purity of the pericyte cultures are confirmed with the expression of classical pericyte markers: NG2, PDGFR-β and CD146. This method demonstrates an efficient and feasible in vitro tool for physiological and pathophysiological studies on pericytes.

**INTRODUCTION:**

Cerebral pericytes are an essential component of the neurovascular unit (NVU), which comprises a functional unit with the cerebral endothelial cells of the blood brain barrier (BBB), glial cells, extracellular matrix and neurons. Pericytes are a vital part in regulated functioning of the central nervous system (CNS) as they serve as one of the interfaces for the exchange of molecular and cellular information.

Cerebral pericytes are embedded in the abluminal side of the brain microvessels, and are essential for establishing[1] and maintaining[2] the BBB physiology. Several recent works have also highlighted the role of cerebral pericytes in angiogenesis[3] and vessel maturation[4], endothelial morphogenesis[5] and survival[6], and in controlling the brain cholesterol metabolism[7]. Importantly, the dysregulation in any of these processes are etiological hallmarks of neurodegenerative diseases.

Indeed, pericytes are a functional necessity for normal BBB functioning and its protection against the progression of several neurological diseases. Degenerating physiology and loss of pericytes are common denominators in the progression of Alzheimer's disease[8], neuronal loss during white matter dysfunction[9], multiple sclerosis[10], septic encephalopathy[11], acute phase ischemic stroke[12] and in other neurological disorders. Pericytes are also instrumental in tumor metastasis[13]. Interestingly, pericytes have also been shown to exhibit a rescuing role after neurological trauma and disorders: in remyelination in brain[1], ischemic stroke, spinal cord injury[14] and promoting angiogenesis[15]. The susceptibility of pericytes to reinforce the pathophysiological manifestation of neurological trauma and disorders makes them a potential therapeutic target[16].

In vitro research models of pericytes in the BBB are important tools to conduct extensive studies. These models provide a platform for more elaborate studies by representing working models of the BBB and more. For instance, these models can be used to understand the cellular physiology within pericytes and among other cell types of the NVU**.** Also, in vitro models are firsthand investigation tools for testing the pharmacological influence of new drugs and molecules on pericytes. These models can also be used to understand the pathophysiological role of pericytes in relation to neurological disorders. Nevertheless, the development of in vitro models requires increased output to enable experimental freedom. These models should be easy and quick, and reduce the number of experimental animals used. In addition, the ability to develop such models into a double and triple cell culture models is desirable.

There are many protocols that have been developed. The protocols proposed by Tigges et al.[17], Chen et al.[18], Thomsen et al.[19], Yamazaki et al.[20], and Crouch and Doetsch[21] are commendable approaches that satisfy most of the necessities. All of these methods yield effective results, but

the dependency on a large number of experimental animals remains a common denominator for these protocols. Therefore, it becomes mandatory to develop a high output method that can isolate and purify pericytes with maximum possible efficiency. In this protocol, the purity of the cells obtained after a second passage is verified with several pericytes markers. We checked for Platelet-Derived Growth Factor Receptor-β (PDGFR- β), which is used as a classical marker of pericytes[17], and for NG2 (neuron-glial antigen 2), which is a marker of pericyte mediated vascular morphogenesis[22] and vascularization[23]. We also checked for cluster of differentiation 146 (CD 146), which has been reported as one of the molecules expressed in the pericytes[17,18].

Here, we present a protocol for the extraction of primary pericytes from mice (wild type or transgenics) that will satisfy all the aforementioned requirements with high output. We employ an antibiotic and immunopanning free selection-based method of proliferation for the primary cerebral pericytes, which will prove itself an efficient model for conducting in vitro studies.

**PROTOCOL:**

All experiments were performed following the Institute's guidelines for the animal use and handling. In accordance with the French legislation, the animal facility at the University of Artois has been approved by the local authorities (reference: B62-498-5). In compliance with the European Union Legislation (Directive 2010/63/EU), all the procedures were approved by the local animal care and use committee (Comité d'Ethique en Expérimentation Animale du Nord-Pas-De-Calais, reference: C2EA 75) and the French Ministry of Research (reference: 2015090115412152).

## 1. Preparation of solutions

1.1. Prepare 500 mL of Washing Buffer A (WBA): 10 mM HEPES solution in Hank's Balanced Salt Solution (HBSS). Store at 4 °C.

1.2. Prepare 500 mL of Washing Buffer B (WBB): 0.1% Bovine Serum Albumin (BSA) with 10 mM HEPES solution in HBSS. Store at 4 °C.

1.3. Prepare 300 mL of 30% dextran solution in WBA by mixing the solution overnight at room temperature. Autoclave the solution at 110 °C for 30 min before use. After autoclaving, let the solution rest at room temperature for 2-3 h. Store the solution at 4 °C.

1.4. Prepare 100 mL of 0.1% BSA solution in cold dextran solution. Shake vigorously for 3-4 min and store at 4 °C.

1.5. Prepare complete Dulbecco's Modified Eagle Medium (DMEM) culture media by dissolving 20% calf serum, 2 mM glutamine, 50 µg/mL gentamycin, 1% vitamins, 2% amino acids Basal Medium Eagle (BME) in Basal DMEM media and store at 4 °C. Add 1 ng/mL Basic fibroblast growth factor (bFGF) prior to use.

1.6. Prepare complete pericyte media by adding pericyte growth supplements (provided with the pericyte media) and 20% Fetal Calf Serum (FCS) in pericyte culture basal media and store at 4 °C.

## 2. Brain tissue recovery and removal of meninges

2.1. For consistency, use mice of similar age and same gender in every batch of extraction. Use a pathogen-free animal shelter and provide ad libitum access to water. To ensure efficiency and minimal use of animals, avoid loss of tissue material.

2.2. Euthanize C57BL/6J, 4-6 weeks old, male mice (Janvier labs, Le Genest-Saint-Isle, France).

2.3. Quickly excise the brain tissue in sterile conditions, avoid any damage to the tissue. Carefully place the tissue in 40 mL of cold phosphate buffered saline (PBS).

2.4. Transfer the brain tissue in cold PBS to a Petri dish (100 mm x 15 mm).

2.5. Place the brain tissue on a sterile dry lint-free wipe and with curved tip forceps, remove the cerebellum, striatum and occipital nerves.

2.5.1. Remove all the visible meninges with a cotton swab. Place the brain tissue upside down and open the lobes with a cotton swab using outward light strokes. Remove all the visible blood vessels.

2.5.2. Place the meninges free brain tissue in a Petri dish (100 mm x 15 mm) with 15 mL of cold WBB.

## 3. Homogenization

3.1. Transfer the tissue to a Dounce tissue grinder mortar tube and then add 3-4 mL of WBB with forceps.

3.2. Mince the tissue with a ‘loose’ pestle 55 times. Rinse the pestle with WBB. Then, mince the slurry with a “tight” pestle 25 times.

3.3. Equally divide the slurry into two 50 mL tubes and add 1.5x volume of cold 30% BSA-dextran, vigorously shaking the tubes to mix the slurry.

## 4. Isolation of the vascular fraction

4.1. After vigorously shaking the tubes, centrifuge the tubes for 25 min at 3,000 x *g* and 4 °C.

4.2. Transfer the supernatant (along with the top myelin layer) to 2 new tubes, and centrifuge the tubes for 25 min at 3,000 x *g* and 4 °C. Preserve the pellets from the first centrifugation by

adding 3 mL of cold WBB (keep the pellet at 4 °C).

4.3. Repeat step 4.2 and carefully preserve the pellets from 2nd centrifugation.

4.4. Discard the dextran and the myelin with tissue debris from the tubes from step 4.3. Preserve the pellets in cold WBB.

4.5. Pool the contents of tube 1 and tube 2 from step 4.1 and make up to final volume of 10 mL with cold WBB.

4.6. Repeat this step for tubes from step 4.2 and step 4.3.

NOTE: Finally, there are 3 tubes from 3 centrifugations.

4.7. Dissociate the pellet with 6 up and down strokes using a 10 mL pipette, until no visible clumps of the pellets are remaining.

4.8. With a vacuum filter assembly and the nylon mesh filter, filter the cell suspensions of each tube.

NOTE: This filtration step is important in order to remove longer/larger vessels via the mesh filter.

4.9. Rinse the first filter in WBB at room temperature in a Petri dish by scraping the filter with a flat tip forceps or scraper. Perform a second filtration with a fresh filter for the rinse to recover more capillaries.

4.10. Divide the filtrate equally to two tubes and centrifuge for 7 min at 1,000 x *g* and RT.

NOTE: During this centrifugation step, prepare the enzymatic solution. Determine the volume of WBB required in accordance with the number of animals used (see **Table of Materials**). Add 1x of DNase 1 and 1x of Tosyl-L-lysyl-chloromethane hydrochloride (TLCK) (see **Table of Materials**) in WBB and pre-warm to 37 °C.

4.11. Collect the pellets from step 4.10 in one tube with pre-warmed WBB with enzymes. Add pre-warmed 1x collagenase/dispase. Place the tube in the shaking table water bath at 37 °C for precisely 33 min.

4.12. Stop the reaction by adding 30 mL of cold WBB. Centrifuge the suspension for 7 min at 1,000 x *g* and RT.

4.13. Discard the supernatant carefully and dissociate the pellet in WBB with 6 up and down strokes using a 10 mL pipette. This step should be less rigorous and comparatively faster.

4.14. Centrifuge the suspension for 7 min at 1,000 x *g* and RT.

NOTE: During this step, discard the coating from the culture dishes and rinse them once with DMEM at room temperature. Coat cell culture dishes for at least 1 h at RT.

4.15. Discard the supernatant from the tube obtained at step 4.14, and dissociate the pellet in new complete DMEM media, plate the cells (Day 0, P0) in 9 wells of 6-well plates (1 well of 9.6 $cm^2$ each).

**5. Proliferation of cerebral pericytes**

5.1 Maintain the cell cultures at 37 °C and 5% $CO_2$ in a sterile incubator. Replace the culture media after 24 h (day 1) of plating the cells by carefully removing the debris. After day 1, change the culture media in every 48 h.

5.2 Observe the cell culture for at least 7-8 days. By this time, cellular growths on the top of endothelial unilayer should be observable.

5.3 Passage the cells on day 8-10 (depending on the confluency) in pericyte culture medium to passage 1 (P1) on gelatin coated culture plates. Change the culture media in every 2 days. Observe the cells for 6-7 days. Cells are consecutively split again to passage 2 (P2) on day 17 [and passage 3 (P3) on day 24 only if required], grown in pericyte medium in gelatin coated plates.

NOTE: Cells shall be ready for experiments/observation at nearly 80-90% confluency.

**REPRESENTATIVE RESULTS:**

This protocol (**Figure 1**) efficiently yields 9 wells (of 6-well plates) at the time of seeding at P0 (**Figure 2A** (P0: Day 1)).

From P0 to P2, there are specific morphological characteristics by which endothelial cells (indicated by white arrows) and s gradual increase in pericytes (indicated by black arrows) can be observed. In P0, the elongated endothelial cells developing from microvessels are in abundance (**Figure 2A**, P0: Day 3), while the abundance of such elongated cells is reduced in P1 and absent in P2. On the contrary, the pericytes appear as quadrilateral cells which are abundant in P2 (**Figure 2A**, P2: Day 18).

To confirm the purity of the pericyte culture in P2, we checked the expression of NG2, CD146 and PDGFR-beta as pericyte markers using quantitative PCR (**Figure 2B**), immunocytochemistry (**Figure 2C**), and western blot (**Figure 3**). Pericytes in P2 express higher levels of CD146, NG2 and PDGFR-β when compared to the expression in total mouse brain (Ms Br) extract. As a control, expression of endothelial markers Occludin and CD31, astrocytes marker Glial Fibrillary Acidic Protein (GFAP) and microglia marker CD11b were also observed absent in P2.

**FIGURE AND TABLE LEGENDS:**

**Figure 1**. **Summary of the protocol.** This outline represents critical steps for pericyte extraction which begins with tissue disintegration with glass pestle grinder followed by 3-step separation in dextran and filtration. This protocol employs a 33 min enzyme digestion step.

**Figure 2. Cells morphology and markers expression.** (**A**) Phase contrast images of pericytes in P0, P1 and P2 stages of proliferation. Abundant endothelial cells in P0 are indicated by white arrows. Their number decreased in P1 and they have disappeared in P2. Pericytes are indicated by black arrows. (**B**) Analysis of CD146, NG2 and PDGFR-β expression by PCR in pericytes in P2 with pericytes in P1 and mouse brain (Ms Br) samples. (**C**) Representative images of pericytes in P2 exhibiting positive immunostaining of CD146, PDGFR-β and, NG2. Scale bar: 50 μm (20x magnification) and 20 μm (40x magnification).

**Figure 3**. **Representative purity of the cell cultures.** Analysis of CD146, PDGFR-β, NG2, Occludin, GFAP, CD31, CD11b and Tubulin expression by western blotting in pericytes in P2 and mouse brain (Ms Br) samples.

**Figure 4**. **Representative comparison of different methods of tissue disintegration, purification, enrichment and enzymatic digestion.** Each published protocol is summarized with indications on the number of animals used for each protocol. Outputs are also indicated with respect to the number of wells obtained upon seeding.

**DISCUSSION:**

Cerebral pericytes are an integral part of the NVU and play an active role in induction and maintenance of the BBB[24]. Similarly, the role of these cells in the different neurodegenerative disorders and vascular pathologies is intriguing. Hence, an efficient high output primary pericyte cell model will provide an efficient platform for in vitro studies.

There are various protocols that have been proposed for the isolation of primary pericytes (**Figure 4**). Tigges et al.[17] suggested a method including cortical tissue with meninges. This approach is tenderization of tissue from 6 mice (a 37 °C, 70 min digestion with papain/DNase enzymes) followed by a disintegration step via 21 G and 18 G needles. This protocol suggests a one-step separation (centrifugation in 22% BSA/PBS solution) that yields at least 2 collagen I coated wells of a 6-well plate. The cells are maintained in endothelial cell growth medium (ECGM) until passage 3 and later in pericyte growth medium (PGM) for passaging cells to promote pericyte proliferation. In another similar approach, Chen et al.[18] proposed tissue dissociation by dicing the tissue with a sterilized razor blade and tissue digestion with collagenase/DNase for 90 min at 37 °C. Following one-step separation (centrifugation in 22% BSA) of the cells, the myelin layer is removed and the pellet is washed twice in ECGM. The microvessels are plated in 3 wells of a collagen I coated 6-well plates. After reaching confluence, the cells are passaged twice and later maintained in PGM. In the end, if cells are passed in ratio of 3, we can obtain 27 wells of 6-well plates only at the use of 10 mice in the beginning of the protocol.

In Thomsen et al., the authors suggest isolation of cerebral pericytes via a two-step enzyme digestion[19]. Meninges and white matter are removed, and brain samples are cut into small pieces.

The tissue pieces undergo the first enzyme reaction in collagenase/DNase I for 75 min at 37 °C, following one step of separation in 20% BSA. The pellet is collected and further digested in collagenase/dispase/DNase I for 50 min at 37 °C. This step is followed by microvessel separation in a 33% Percoll gradient and further washed once. The microvessels are seeded on collagen IV/fibronectin coated 35 mm dishes. The proliferation of pericytes is favored by 10% FCS and gentamicin sulphate in DMEM for 10 days. In another two-step enzyme digestion approach, Yamazaki et al. suggest mincing of the excised tissue in cold DMEM[20]. In the first enzyme reaction, samples are treated with collagenase/DNase I for 75 min at 37 °C. Following one step centrifugation, the pellet is again washed once and a second enzyme reaction is initiated in collagenase/dispase for 60 min at 37 °C. Following a one-step separation, the pellet is resuspended and centrifuged in 22% BSA solution. Finally, the microvascular pellet is resuspended and plated in a 6-well plate. For 5 mouse brains, 1 well of a 6-well plate can be plated. To obtain the pericytes, endothelial cultures are passaged thrice while maintained in mouse brain endothelial cell (mBEC) medium II. Crouch and Doetsch[20] suggest pericyte purification method by FACS. Tissue samples from cortex and ventricular–subventricular zone of mouse brain are micro-dissected and minced thoroughly with a scalpel. After collagenase/dispase enzyme incubation for 30 min at 37 °C, the digested tissue is separated from myelin and debris centrifuged in a 22% v/v Percoll solution. The cell suspension is then incubated in fluorescently conjugated antibodies for FACS analysis and sorting. The sorted cells are plated in collagen coated wells of 24 well plate. It is suggested that one cortex yields enough cells for one plating in 1 well of 24-well plate.

Even if productive, these methods come with several limitations, from the usage of high number of animals for single batch isolation to a very limited amount of output.

During the development of this proposed protocol, we were successful in obtaining high output: 9 wells of a 6-well plate from as few as 10 mice. To this end, removal of meninges ensures the one step removal of large vessels from the tissue. The Dounce tissue grinder is more appropriate for soft tissues such as the brain. It also ensures sample reduction with the loose pestle, homogenization with the tight pestle, and prevents unnecessary cellular damage. One of the main objectives in primary cell culture protocols is the minimal waste of tissue and extended retrieval of cerebral vasculature. In the presented protocol, this is achieved by repetitive centrifugation of the dextran-BSA infused tissue homogenate. A three-step centrifugation approach helps to recover large quantities of vasculature from the tissue homogenate. This provides a 3x enhanced recovery of microvessels. Following separation, filtration is the next essential step, which favors the exclusion of smooth muscle cell associated large vessels. As mentioned before a combination of different enzymes has been proposed for enzymatic digestion. While DNase and collagenase/dispase are used to reduce clumps of cells and isolate single cells respectively, it is very important to prevent cell death in such an invasive environment and this is prevented by TLCK, which thereby increases the final yield. Initially, the first passage is allowed to grow an endothelial monolayer, which later supports the growth of attached pericytes on the unilayer. Since the survival of primary endothelial cells is reduced upon passaging, it enhances the probability for pericyte retrieval. Moreover, this protocol employs another passaging that ensures avoidance of endothelial cell contamination. It should be noted

that with a higher number of cells from P2, the dependency on further passaging of the cells is reduced. In addition, it reduces the possibility of pericyte growth being overtaken by smooth muscle cells, which proliferate on much higher rate.

In order to achieve a higher output, there are several steps that are critical and should be accurately performed with respect to temperature and time. The mixing of tissue homogenate into BSA-dextran should be fast. The pellet dissociation after the centrifugation steps should be quick to prevent cell death. Moreover, 33 min of enzymatic digestion should be done with precision and care. One of the limitations for this protocol is the 7-8 day duration that endothelial unilayer is allowed to grow and further facilitate the growth of pericytes. Evidently, the isolation of microvessels is btter, the growth of unilayer is faster, and hence there are an increased number of pericytes. It is recommended not to use less than 10 mice in each extraction to ensure an adequate number of microvascular fractions to support the pericyte growth further. If the aforementioned points are followed carefully, the desired cell density for cerebral pericyte culture can be easily achieved.

In vitro models provide a feasible platform for the development of derivative models to obtain more information on the pathophysiological relevance and communication among the other cells of the NVU during neurological disorders. Isolated pericytes can be incorporated in a bi- cellular culture (with endothelial or glial cells) and tri-cellular culture (endothelial and glial cells) models. The development of these models has not been discussed here. To conclude, this protocol provides one approach for the isolation of primary cells with higher output and a better platform for in vitro research related to the cerebral pericyte biology.

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**DISCLOSURES:**
The authors have nothing to disclose.

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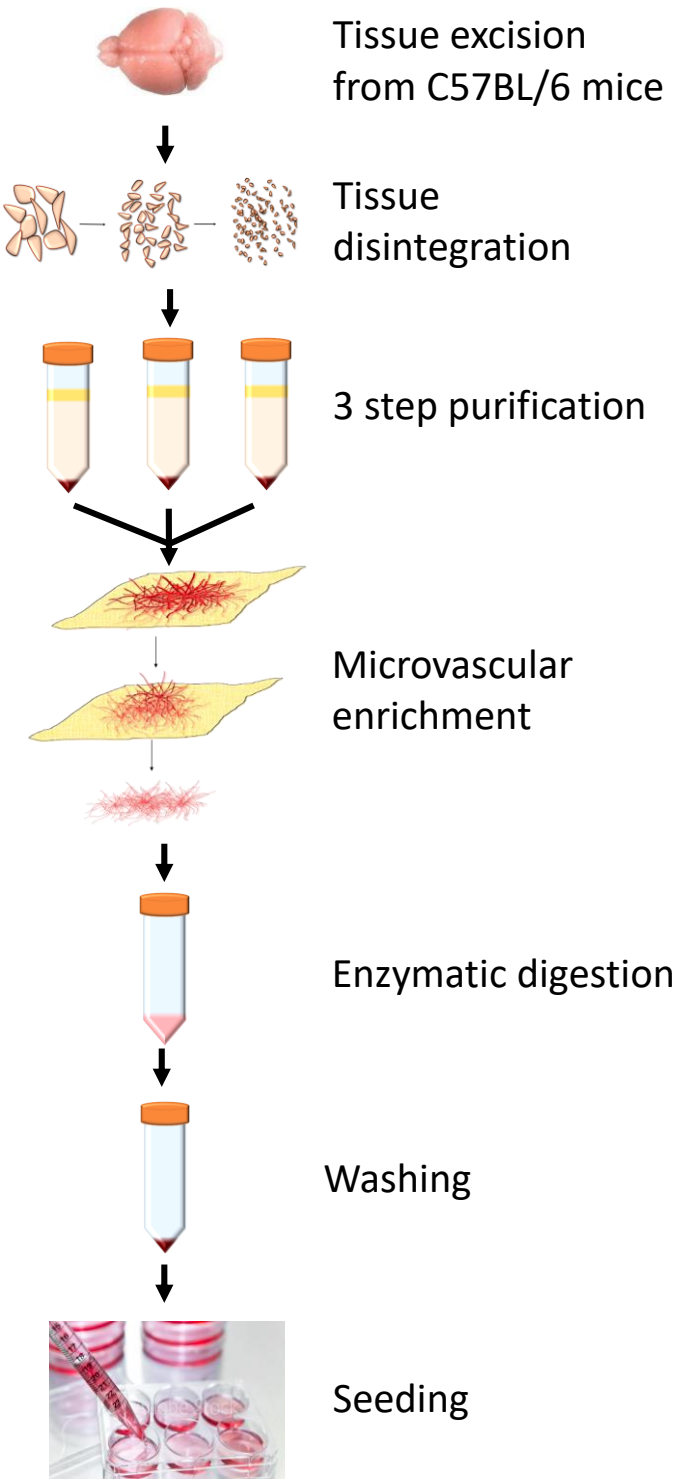
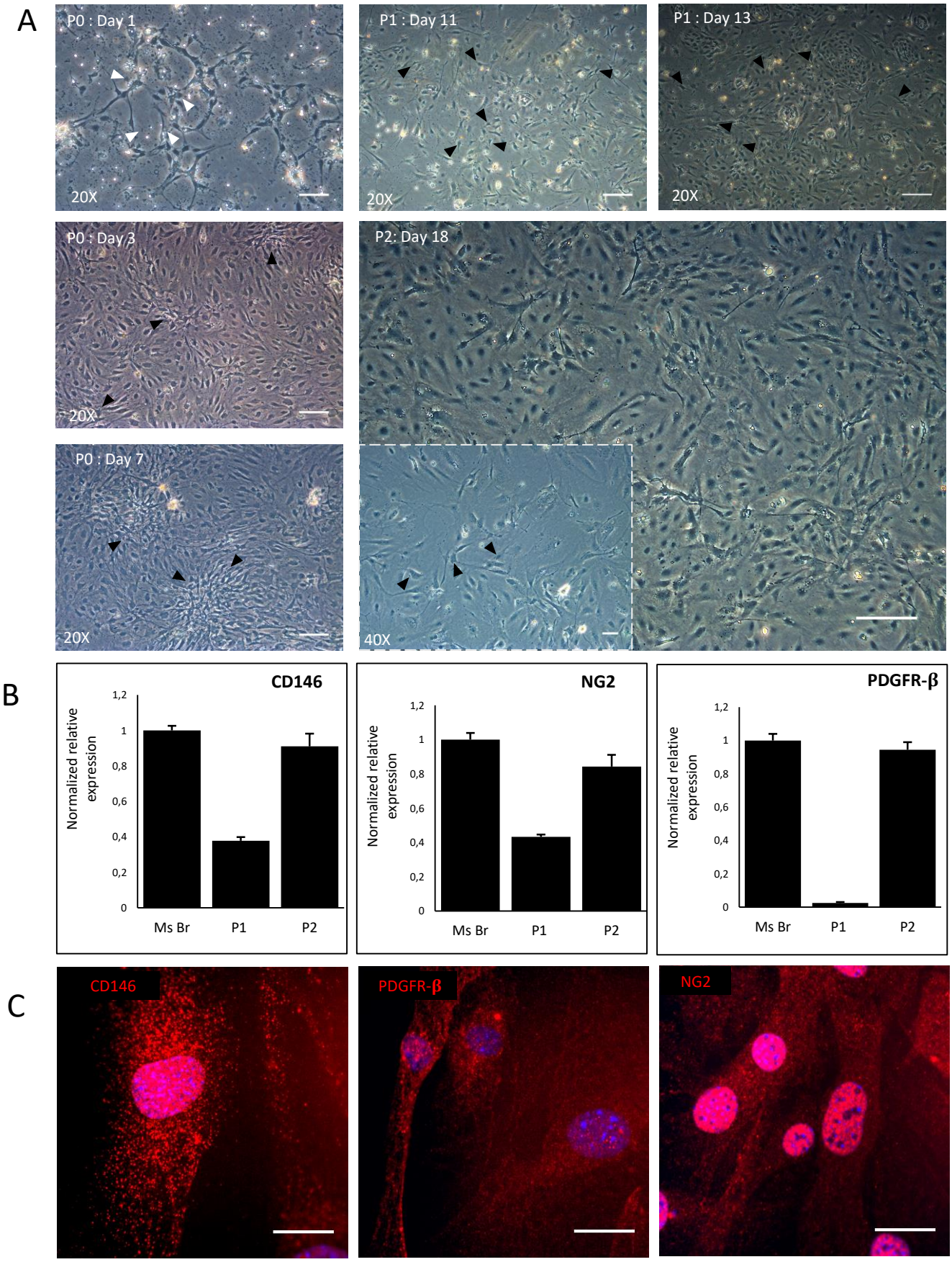
Figure





Figure





Figure

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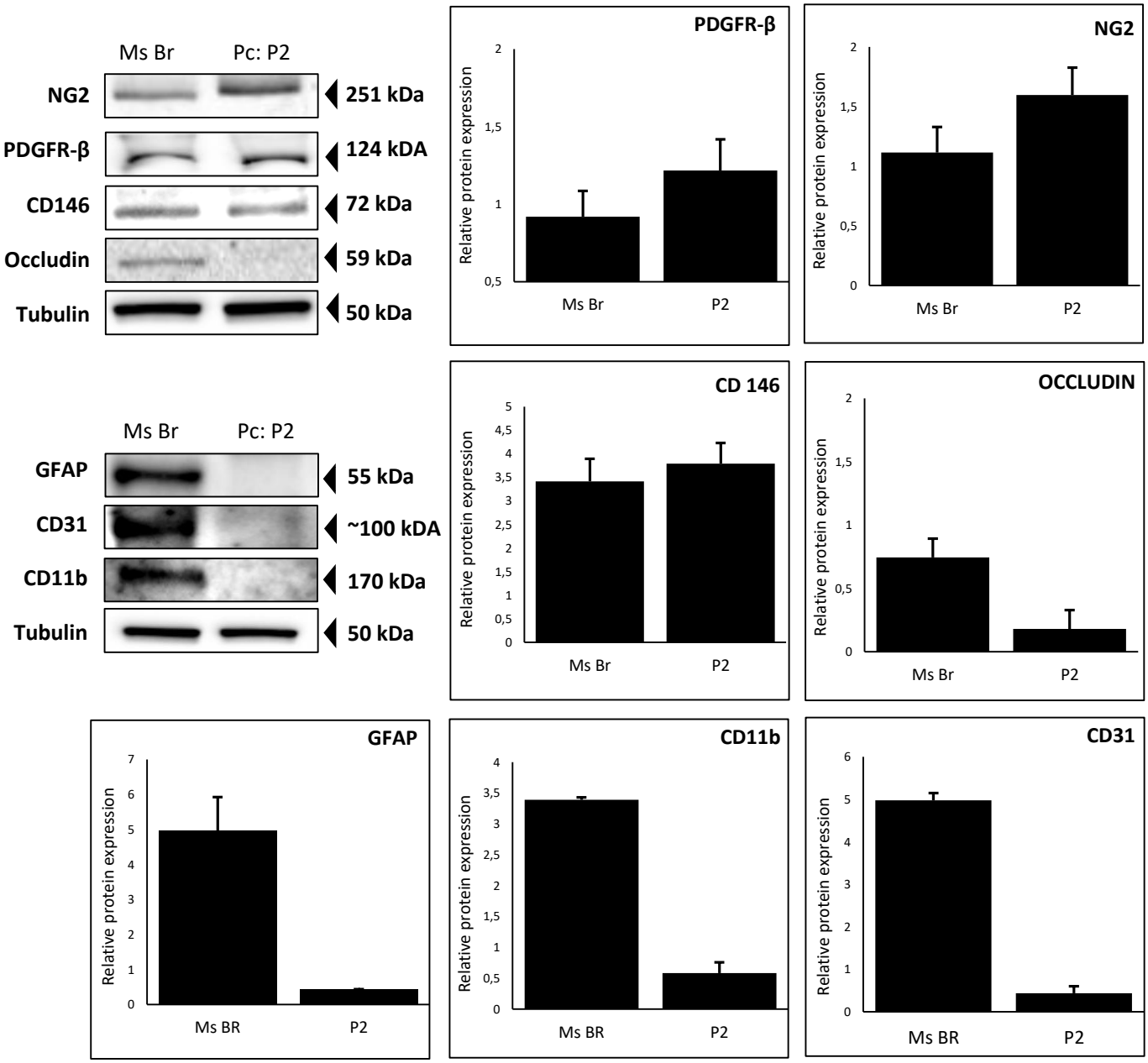

Figure



| | Our protocol | Tigges *et al.* | Chen *et al.* | Crouch and Doetsch | Thomsen *et al.* | Yamazaki *et al.* |
|---|---|---|---|---|---|---|
| Treatment of tissue  | - meninges /white matter | No | - meninges /white matter | No | - meninges /white matter | - meninges /white matter |
| Tissue disintegration  | Dounce tissue grinder | 21/18 gauge needle | Razor blade | Scalpel | Scalpel | - |
| Purification  | 3X/ BSA Dextran | 1X/ BSA PBS | 1X/BSA PBS | 1X/Percoll | 1X/ Percoll | 1X/ BSA PBS |
| Enrichment  | 2X filtration/59µm | No | No | FACS | No | No |
| Enzymatic digestion  | 1X/33’ | 1X/70’ | 2X/90’-60’ | 1X/30’ | 2X/75’-50’ | 2X/75’-60’ |
| Animal expense  | 10 / 9 wells of 6 well plate | 6 /2 wells of 6 well plate | ? /3 wells of 6 well plate | 1 /1 well of 24 well plate | ? /? wells of 35 mm plate | 5 /1 well of 6 well plate |

| Material Requirements | | | |
|---|---|---|---|
| **Title** | **Company** | **Catalog Number** | **Comments/Description** |
| Amino acids BME | Sigma | B-6766 | Store at 4 °C. |
| Basal DMEM media | Invitrogen | 316000083 | Store at 4 °C. |
| Basic fibroblast growth factor | Sigma | F-0291 | Store at -20 °C. |
| BSA | Sigma | A-8412 | Store at 4 °C. |
| Collagenase dispase | Sigma | 10269638001 | Prepare a 10x stock solution in sterile PBS-CMF. Filter the solution with a 0.22 µm syringe filter and store at -20 °C. Note: For the enzyme digestion step of the protocol, for every set of 10 mice for extraction, 300 µL of 10x collagenase dispase is required. |
| Dextran | Sigma | 31398 | |
| DNase I | Sigma | 11284932001 | Prepare a 1000X stock solution by dissolving 100 mg in 10 ml sterile water and store at -20°C. |
| Gelatin | Sigma | G-2500 | Prepare the working coating by making a 0.2% gelatin solution in sterile PBS-CMF (8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L $KH_2PO_4$, 2.86 g/L $NaHPO_4$ (12 $H_2O$), pH 7.4). Autoclave the solution for minimum 20 minutes at 120 °C and store at room temperature. Culture dishes to be coated for at least 4 hours at 4 °C. |
| Gentamycin | Biochrom AG | A-2712 | Store at 4 °C. |

| | | | |
|---|---|---|---|
| Glutamine | Merck | I.00289 | Store at -20 °C. |
| HBSS | Sigma | H-8264 | Store at 4 °C. |
| HEPES | Sigma | H-0887 | Store at 4 °C. |
| Matrigel | BD Biocoat | 354230 | Prepare a working coating solution of Matrigel by diluting stock in cold DMEM at 1:48 ratio with its final concentration to be 85 µg/cm$^2$. Cell culture dishes should be coated at least for 1 hour at room temperature. |
| Pericyte Medium-mouse | Sciencell research laboratories | 1231 | Store at 4 °C. |
| )syl Lysin Chloromethyl Keto | Sigma | T-7254 | Prepare a 1000X stock solution in WBA by dissolving 16 mg in 10.88 mL of WBA to make a 4 mM solution and store at 4 °C. |
| Vitamins | Sigma | B-6891 | Store at -20 °C. |

## Equipment Requirements

| | |
|---|---|
| Filtration tools | Sefar, Nylon mesh, 60-micron porosity |
| Laboratory equipment | Swing bucket rotor centrifuge<br>Water bath with agitator<br>Laminar Flow Hood : BSL2 |
| Glassware (all components to be heat sterilized) | Dounce Tissue Grinder With Glass Pestle<br>Pestle I: 0.0035 - 0.0065 inches<br>Pestle II: 0.0010 - 0.0030 inches<br>Vacuum filter assembly with coarse porosity fritted glass filter support base |

| | |
|---|---|
| Surgical dissection tools (all components to be heat sterilized) | Forceps, scissors, Bunsen burner, cotton swabs, gauge |

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| | |
|---|---|
| Title of Article: | An easy and efficient high output method to isolate brain pericytes from mouse |
| Author(s): | Anupriya Mehra, Lucie Dehouck, Elodie Vandenhaute, Marc Fatar, Laurence Fenart, Fabien Gosselet |

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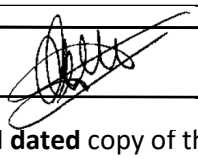
| Signature: |  |
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Submission of our revised manuscript JoVE 60588

Dear Alisha Dsouza,

In reference to your E-mail of September 06th 2019, please find enclosed our revised manuscript entitled « An easy and efficient high output method to isolate cerebral pericytes from mouse».

We thank the 4 reviewers for their productive criticism and helpful comments. We have made every attempt to address the concerns raised by the reviewers. New experiments have been performed and western blots have been added. Our responses to their suggested revisions are detailed in our point-by-point reply below. Please find the original remarks by the reviewers written in italic letters followed by our reply written in blue and upright letters.

We are convinced that our study is significantly improved by following the reviewers suggestions. We hope to have addressed all comments in a satisfactory manner to allow for publication of our revised manuscript in the JoVE Journal.

At last, please note that we also considered the editorial comments and have modified accordingly.

Yours sincerely,

Fabien Gosselet

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Comments from Peer-Reviewers

**Reviewers' comments:**

**Reviewer #1:**

*Major Concerns: The efficiency in terms of yield of pericytes as compared to other published alternative protocols has not been demonstrated. This would require a significant additional amount of work but it would most definitely increase the value and impact of the current study significantly.*

We totally agree with the reviewer #1 that the comparison of efficiency in terms of yield of pericytes is only based on values and results claimed by authors in their original publications. The proposition of the reviewer to realize these 6 protocols (because 2 new protocols have been introduced in our discussion) in parallel to directly compare the different yields is an interesting suggestion but would require a huge amount of work as well as the use of several materials and equipment that we do not possess (FACs, etc). The objective of our study was to develop a cheap, fast and efficient method using traditional equipment easily found in all laboratories (centrifuge, etc). And we did it. Therefore we prefer do not follow this suggestion.

*Minor Concerns:Language revision is warranted*

Addressed. Manuscript has been deeply checked for language revision.

**Reviewer #2:**

*Major Concerns: The authors have performed staining and immunoblotting (Figure 2) showing expression of NG2, PDGFRβ, and CD146 in isolated pericytes. These markers are mural cell markers but not 100% specific to the pericyte sub-population. A single-cell RNA seq analysis published last year showed that Pdgfrb and Cspg4 are expressed in both pericytes and smooth muscle cells (SMCs) but also in fibroblasts (FBs) and oligodendrocyte progenitor cells (OPCs) (Nature 2018; 554:475-480). Also, looking at Mcam gene (i.e., CD146 protein), the highest levels are found in SMCs and a little in oligodendrocytes. Even though the authors use filtration steps, it remains uncertain whether contamination from other cell types - namely, SMCs and FBs - may occur. Additionally, both the grey and white matters are taken into account which may increase contamination issues (e.g., myelin debris). An additional bulk RNAseq experiment could be performed and compared to the current Betsholtz database in order to reinforce the proposed protocol. No one will question the purity if this can be shown.*

To address the question of brain pericytes purity raised by the reviewers #2, #3 and #4, we performed additional western blots using specific antibodies directed against CD31, GFAP and CD11b that specifically target endothelial cells, astrocytes and microglial cells, respectively. These new results have been included in the figure 3 and clearly demonstrate that there is no contamination by these different cell types in our final cultures. Therefore, new experiments have been done, a new figure has been added. All these results have been analyzed and discussed in the appropriate section of the manuscript.

*- Authors should provide a picture of their initial preparation prior dissociation steps. This image should show vessels only and would be the first image of Figure 2A.*

Dissociation steps often occur in our protocol, therefore we do not exactly understand which step is concerned by the reviewer's suggestion. However, as a JoVE protocol, our method will be translated into a movie and we are confident with the fact that all the mandatory steps will be filmed.

*- Figure 2D: It is very surprising to see that PDGFRβ levels are equal in mouse brain vs pericyte samples (see Supplemental Figure 1a, Nat Neurosci. 2019; 22:1089-1098 and Figure 1, Neurosci Lett. 2015; 607:97-101)*

We thank the reviewer for this comment. Indeed, as in the both studies cited above, we clearly demonstrate that brain pericytes express high level of PDGFR-β. In addition, these both studies claim that brain pericytes express higher levels of PDGFR-β than endothelial cells and vascular smooth muscle cells. In our study, we demonstrate that cerebral pericytes express equally PDGFR-β than total mouse brain. Interestingly, this receptor has also been reported highly expressed by neurons (J Cereb Blood Flow Metab. 2012, 32(2):353-367). Therefore, from our point of view, this is the explanation why we observe a high level of this receptor in total mouse brain extract that include the protein content from all the cell types (i.e. ECs, OPCs, vSMCs, neurons and pericytes, etc).

*- Also, it is unclear why the authors decided to compare pericyte protein profiles with the total mouse brain. One would think that comparing pericytes to endothelial cells and/or commercially available pericytes from ScienCell (to name one) would be more informative and would definitely strengthen the manuscript in my opinion. Using the total mouse brain as comparison will only bring confusion to the readers.*

The objective of our study was to demonstrate that our protocol and method are efficient to purify mouse brain pericytes from a total mouse brain. For this reason, we used the total mouse brain extract to compare the mRNA and protein expression levels with cultures of brain pericytes. Following reviewers'suggestions, we analyzed expression of specific markers of endothelial cells, microglial cells and astrocytes in our culture of brain pericytes and in total mouse brain fraction. We clearly demonstrate that the level of these markers decrease after extraction, purification and cultivation of these cells. In parallel, the expression of specific markers of brain pericytes is stable or increased (Figures 2B, 2C and 3). In addition, these cells show a specific morphology, as demonstrated by figure 2A.

Then, comparison of these cells with brain pericytes provided by ScienceCell is a very interesting suggestion, but we consider that there is an evident lack of evidence that these cells are really brain pericytes. There is no information on the extraction/purification protocol. In addition, these cells are not well characterized and only very few information are available on the website. It is mentioned that they express PDFGR-β and αSMA, but there is no evidence that they express other pericytes markers. Therefore, we consider that it would be not relevant to compare our cell culture with these cells.

*Minor Concerns: It would be of interest to have a native English speaker checking the present manuscript before eventual resubmission.*

Addressed. As suggested by the reviewer, the manuscript has been deeply checked for language revision.

**Reviewer #3:**

*Major Concerns: The purity of the cell culture is not clear. The extracts will invariably contain brain endothelial cells and the authors need to demonstrate that pollution from at least these cells. There could also be pollution from macrophages and astrocytes. It would certainly be more convincing with double-labelings of hall-mark markers for endothelial cells, astrocytes, and microglia like CD31, GFAP and CD11b, and preferably in larger mags as this will convince the reader better about the purity of the pericyte cultures.*

We thank the reviewer for this suggestion. Following this recommendation, we performed additional western blot experiments using specific antibodies against CD31, GFAP and CD11b that specifically target endothelial cells, astrocytes and microglial cells respectively. These new results have been included in the figure 3 and clearly demonstrate that our primary cultures are not contaminated by these different cell types. Results and discussion parts have been updated accordingly.

*The yield may be high, but the authors are not quoting all papers that already set up protocol, e.g. disappointingly the authors fail to quote important papers (e.g. Thomsen et al, PMID: Thomsen MS et al. 27456748; Yamazaki Y et al. 26883501) in this context which also demonstrate high yield in their isolation of primary brain pericytes with virtually identical yield.*

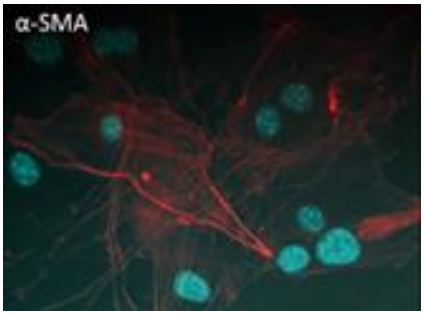
Following the reviewer's suggestion, we added and discussed these interesting references in the manuscript. However, we decided to do not include one of the reference of Thomsen et al., that describes brain pericytes purification using brain from pigs. The objective of our work being to compare the "mouse" protocols together, we decided that it would be not relevant to include data and methods obtained in porcine. Hope that the reviewer will understand our decision.

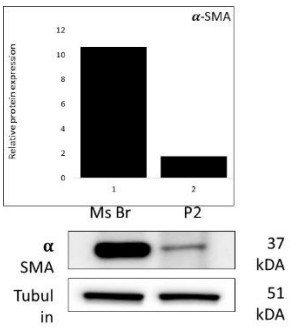
*The functional characteristics of the isolated pericytes should be further explored. Did the pericytes enable the authors to co-culture the brain endothelial cells to improve the TEER and lower passive permeability ?*

We thank the reviewer for this interesting suggestion and totally agree that functional characteristics of brain pericytes merit to be further investigated. For sure, influence of brain pericytes on brain endothelial cells physiology and monolayer integrity is an interesting point that we would like to investigate in our next studies. Indeed, objective of our study was to develop a fast and efficient method to obtain pure cultures of cerebral pericytes from mouse. But it was not for investigating influence of brain pericytes on blood-brain barrier physiology. However, following reviewer's suggestions, we included in the discussion part the fact that these primary cerebral pericytes might be used in such studies.

*When cultured in 20 % FCS, will this change the phonotype of the pericytes ? Did the authors look for alpha-SMA expression?*

When cultures in 20% FCS, morphology of brain pericytes is still normal with no visible change in phenotype. In culture conditions, they normally attain a tetrahedron like shape. To answer the second question, we checked for alpha-SMA expression, which can be stated as minimal expression when compared with mouse brain extract that is consistent with an undifferenciated state of brain pericytes as demonstrated previously (Tigges et al. and Dellavalle et al).




Left : IF of alpha-SMA in our cell culture. Right : Western blot showing low expression of alpha-SMA in total mouse brain compared to brain pericytes.

*Minor Concerns: Abstact: …."we propose", use another verb. No indications of arrows in Fig.2*

Addressed. This sentence has been changed. Arrows are now explained in the representative results section and in figures legend.

**Reviewer #4:** Manuscript Summary:

*Major Concerns: One potential major concern of this protocol is that the authors did not address how to avoid the contamination of astrocytes. It would be highly recommended to check whether the authors' pericyte cultures would not be contaminated with astrocytes.*

We thank the reviewer for this helpful comment. To demonstrate the absence of astrocytes contamination into our cell cultures, we tested the expression of the GFAP marker, specific of this cell type. Results are shown in Figure 3 and demonstrate that our protocol is efficient to decrease the expression of GFAP which is absent in our culture, but highly expressed in brain total fraction. In addition, we also investigated the contamination by microglial cells and endothelial cells. Again, our results show that there is no contamination.

Minor Concerns: Figure 2: the authors need to use the term "PDGF-R-beta" instead of "PDGF-R".

Addressed. We remplaced PDGF-R by PDGFR-β.