A High Output Method to Isolate Cerebral Pericytes from Mouse

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TITLE:

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

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SUMMARY:

We have developed a new protocol for the extraction of murine cerebral pericytes with a high output result. This method is based on an antibiotic-free enrichment oriented pericyte extraction. This protocol represents a valuable tool for *in vitro* studies providing high purity and high yield, thus allowing to decrease number of experimental animals.

ABSTRACT:

In recent years cerebral pericytes have become the focus of extensive research in the field of vascular biology and pathology. The importance of pericytes in blood brain barrier formation and physiology is now demonstrated but molecular basis remains largely unknown. The pathophysiological role of cerebral pericytes in neurological disorders is intriguing and of great importance. For this purpose, the *in vitro* models are not only sufficiently appropriate but also feasible to incorporate different techniques for these studies. Needless to say, several methods have been proposed as *in vitro* models for the extraction of cerebral pericytes, although an antibiotic-free protocol with high output is always desirable. Most importantly, a method which has higher productivity per extraction reduces the dependency on usage of more animals.

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Here we propose a simple and efficient method for extracting cerebral pericytes with sufficiently high productivity. In this protocol, the mouse brain tissue homogenate is mixed with 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 microvessels rich filtrate.

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

INTRODUCTION:

Cerebral pericytes are an essential component of 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 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 of the brain cholesterol metabolism[7]. Importantly, the dysregulation in any of these processes are etiological hallmarks of neurodegeneratives diseases.

Indeed, pericytes are a functional necessity for a normal BBB functioning and its protection against the progression of several neurological diseases. Degenerating physiology and loss of pericytes are common denominators in 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 the tumour metastasis[13]. Interestingly, pericytes have also been shown to exhibit a rescuing role after the neurological trauma and disorders, such as 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 focus to the importance of pericytes in the BBB, an *in vitro* research models are an important tool to conduct extensive studies. These models provide a platform for more elaborative studies *via* representing as 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 first hand 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 with neurological disorders. Nevertheless this should be duly noted that the development of *in vitro* models requires a higher output to enable the experimental freedom. These models should be easy, quick, and reduce the dependency on usage of higher number of experimental animals. In addition, the feasibility of such models to be developed into a double and triple cells culture models is desirable.

Needless to say, there are many protocols that have been developed. The protocols proposed before by Tigges *et al*.[17], Chen *et al*.[18], Thomsen *et al.*[19], Yamazaki *et al.*[20], and Crouch and Doetsch[21] propose commendable approaches which suffice most of the necessities. These all methods propose feasible protocols with effective results, although the dependency on the usage of large number of experimental animals yet remains a common denominator for these protocols. Therefore, it becomes mandatory to develop a new high output methods allowing to

isolate and purify pericytes yet with maximum possible efficiency. In this protocol, the purity of the cells obtained after second passage is checked with several markers of pericytes. For instance, we checked Platelet-Derived Growth Factor Receptor-β (PDGFR-β) which is used as a classical marker of pericytes[17]. In addition, we checked NG2 (neuron-glial antigen 2) as the marker of pericyte mediated vascular morphogenesis[22] and vascularization[23] . We also checked cluster of differentiation 146 (CD 146) which has been reported as one of the molecules expressed in the pericytes[17,18].

With this article, we present a new protocol for the extraction of primary pericytes from mice (wild type or transgenics) which will suffice all the aforementioned requirements with considerably high outcome. 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:

1. Preparation of solutions

1.1 Buffers and solutions

1.1.1 Washing Buffer A (WBA)

Prepare a 10 mM Hepes solution in Hank's Balanced Salt Solution (HBSS) and store at 4°C.

NOTE: Recommended final volume of this solution is 500 ml.

1.1.2 Washing Buffer B (WBB)

Prepare a 0.1% Bovine Serum Albumin (BSA) with 10 mM Hepes solution in HBSS and store at 4°C.

NOTE: Recommended final volume of this solution is 500 ml.

1.1.3 Dextran solution

Prepare a 30% dextran solution in WBA by mixing the solution overnight at room temperature. Autoclave the solution at 110°C for 30 minutes before use. After autoclave let the solution rest at room temperature for 2-3 hours. Store the solution at 4°C.

NOTE: Recommended final volume of this solution is 300 ml.

1.1.4 Dextran BSA solution

Prepare a 0.1% BSA solution in cold dextran solution followed by shaking vigorously for 3-4 minutes and store at 4°C.

NOTE: Recommended final volume of this solution is 100 ml.

1.2 Culture media

1.2.1 Complete Dulbecco's Modified Eagle Medium (DMEM) culture media.

Prepare complete 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.

1.2.2 Pericyte medium.

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.

General comments for mouse experiment

- All experiments are required to be performed with 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)
- To obtain consistency in results, it is recommended to utilize the mice of similar in age and gender in every batch of extraction.
- Animal shelter should be pathogen free and provided with adequate water ad libitum.
- To ensure efficiency and minimal use of animals, loss of tissue material should be avoided.

2. Brain tissue recovery and removal of meninges

2.1 Euthanize C57BL6J, 4-6 weeks old, male mice (Janvier labs, Le Genest-Saint-Isle, France).

2.2 Perform a quick excision of the brain tissue in sterile conditions, avoid any damage to the tissue. Carefully place the tissue in 40 ml of cold PBS (Phoshate buffer saline).

2.3 Transfer the brain samples in cold PBS in a petri dish (100 mm x 15 mm).

2.4 Place the tissue on a sterile dry lint and with the help of curved tip forceps remove the cerebellum, striatum and occipital nerves. Following this, remove all the visible meninges with the help of a cotton swab. Place the brain upside down and open the lobes with the help of cotton swab doing outward light strokes. Remove all the visible blood vessels. Place the meninges free brain tissue in a petri dish (100 mm x 15 mm) with 15 ml cold WBB.

3. Homogenization

3.1 Transfer the tissue in a dounce tissue grinder mortar tube followed by adding 3-4 ml of WBB with the help of forceps.

3.2 Mince the tissue with the 'loose' pestle for 55 times. Rinse the pestle with WBB. Finally mince the slurry with "tight" pestle for 25 times.

3.3 Transfer the slurry in two 50 ml falcons equally and add 1.5 times part volume of cold 30%

BSA-dextran, vigorously shaking the tubes to mix the slurry in dextran.

4. Isolation of the vascular fraction

4.1 While vigorously shaking the tubes, put them for the 1st centrifugation at 3000 g for 25 minutes at 4°C.

4.2 Transfer the supernatant (along with the top myelin layer) into 2 fresh tubes, repeat the previous step (4.1). Reserve the pellets from the first centrifuge by adding 3 ml of cold WBB (keep the pellet at 4°C).

4.3 Repeat step 4.2, followed by step 4.1 and carefully preserving the pellets from 2nd centrifuge.

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

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

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

NOTE: Finally, you obtain 3 tubes from 3 centrifugations.

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

4.8 With the help of 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* mesh filter.

4.9 Rinse the 1st filter in WBB at room temperature in a Petri dish by scraping the filter with a flat tip forceps or scraper. Perform a 2nd filtration with a fresh filter for the rinse to recover more capillaries.

4.10 Separate the filtrate in two equal parts and centrifuge at 1000 g for 7 minutes at room temperature.

NOTE: During this centrifugation step, the enzymatic solution has to be prepared. 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.

4.11 Pool down the pellets from step 4.10 in one tube in pre-warmed WBB with enzymes.

Finally, add pre-warmed 1x collagenase dispase. Place the tube in the shaking table water bath until the end of enzyme digestion.

NOTE: This digestion step consists to incubate tube for very precisely 33 minutes at 37°C.

4.12 Stop enzyme reaction by adding 30 ml of cold WBB. Centrifuge the suspension at 1000 g for 7 minutes at room temperature.

4.13 Discard the supernatant carefully and dissociate the pellet in WBB with the help of 6 up and down strokes using a 10-ml pipette.

NOTE: This step should be less rigorous and comparatively faster.

4.14 Centrifuge the suspension at 1000 g for 7 minutes at room temperature.

NOTE: During this step, discard the coating from the culture dishes and rinse them once with DMEM at room temperature. Cell culture dishes should be coated at least for 1 hour at room temperature.

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 hours (day 1) of plating the cells with carefully removing the debris. After day 1, change the culture media in every 48 hours.

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

5.4 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:

In current status this protocol (summarized in **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 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**), western blot (**Figure 3**) and immunocytochemistry (**Figure 2C**). Pericytes in P2 express higher levels of CD146, NG2 and PDGFR-beta 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-minute 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 decrease 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.

Table 1. **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. For instance, Tigges *et al*. [17] suggest a method including cortical tissue with meninges. This approach is tenderization of tissue from 6 mice (a 37°C, 70 minutes digestion with papain/DNase enzymes) followed by a disintegration step *via* 21 and 18 gauge needles. This protocol suggests a one-step separation (centrifugation in 22% BSA/PBS solution) which shall yield 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] propose the tissue dissociation with dicing the tissue with a sterilized razor blade and tissue digestion with collagenase/DNase for 90 minutes 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 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 minutes 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 minutes at 37°C. This step is followed by microvessel separation in 33% Percoll gradient and further washed once. The microvessels are seeded on the collagen IV/fibronectin coated 35 mm dishes. The proliferation of pericytes is favoured 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 minutes at 37°C. Followed by one step centrifugation, the pellet is again washed once and second enzyme reaction is initiated in collagenase/dispase for 60 minutes 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 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 mins at 37°C. Subsequently, the digested tissue mince is separated from myelin and debris centrifuged in 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 one well of 24-well plate.

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

During the development of this proposed protocol, we are successful in obtaining high output i.e. 9 wells of 6 wells plate from as few as 10 mice. To this end, removal of meninges ensures the first 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 loose and 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 yet extended retrieval of cerebral vasculature. In our proposed protocol, this is achieved *via* 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 three times enhanced recovery of microvessels. Following separation, filtration is the next essential step which favours the exclusion of smooth muscle cells associated large vessels. As mentioned before a combination of different enzymes has been proposed for the 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 endothelial monolayer, which later supports growth of attaching pericytes on the unilayer. Since the survival ability of primary endothelial cells is reduced upon passaging, it enhances the probability for retrieval of pericytes. Moreover, this protocol employs another passaging which ensures avoidance of endothelial cells contamination. This should also be noticed that with the benefit of higher number of cells as output in P2, the dependency on further passaging of the cells is reduced. In addition, it reduces the possibility of pericyte growth overtaken by smooth muscle cells which proliferate on much higher rate.

In order to achieve a higher output, there are several steps which are critical and should be accurately performed with respect to temperature and time. For instance, 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, the duration of 33 minutes of enzymatic digestion should be done with precision and care. One of the limitations that can be listed for this protocol is the duration of 7-8 days for which endothelial unilayer is allowed to grow and further facilitate the growth of pericytes. Evidently, better is the isolation of microvessels, faster is the growth of unilayer, and hence an increased number of pericytes. It is also recommended not to use less than 10 mice in each extraction so as to ensure an adequate number of microvascular fractions to support the pericytes growth further. If aforementioned points are followed carefully, the desired cell density for cerebral pericyte culture can be easily achieved.

As mentioned before that *in vitro* models shall provide a feasible platform for the development of derivative models to provide 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- (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, we hope this protocol will provide a newer approach for isolation of primary cells to aim a higher output and will serve a better platform for *in vitro* research related to the cerebral pericytes biology.

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DISCLOSURES:

The authors have nothing to disclose.

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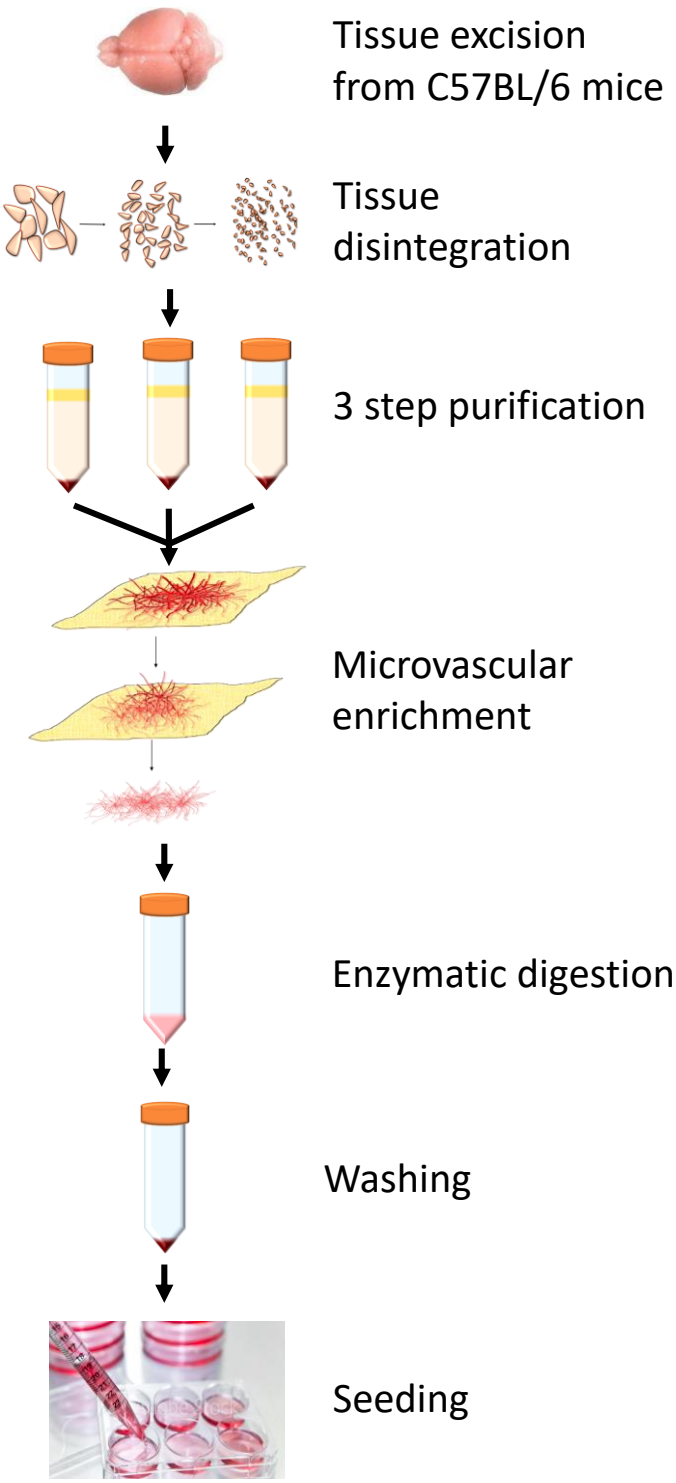

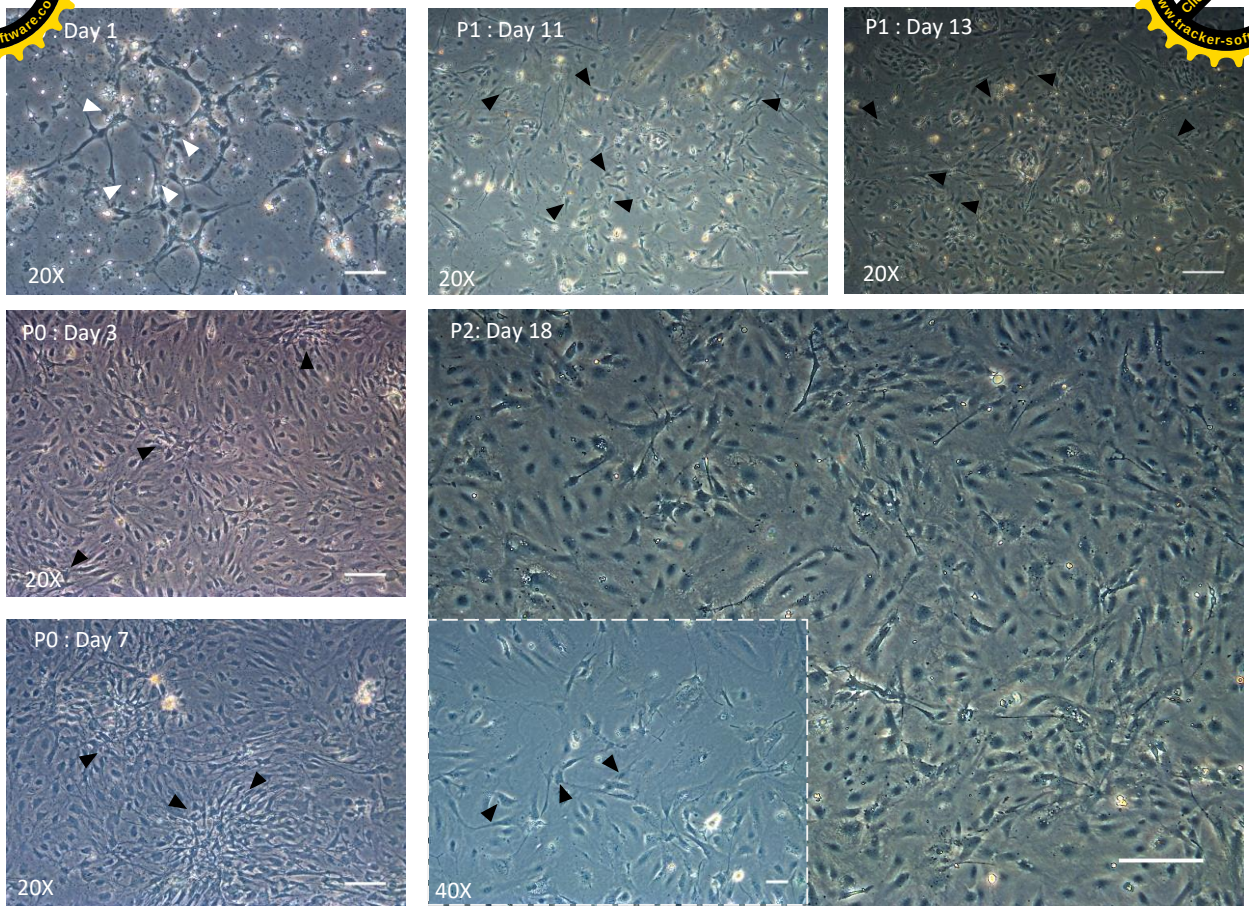

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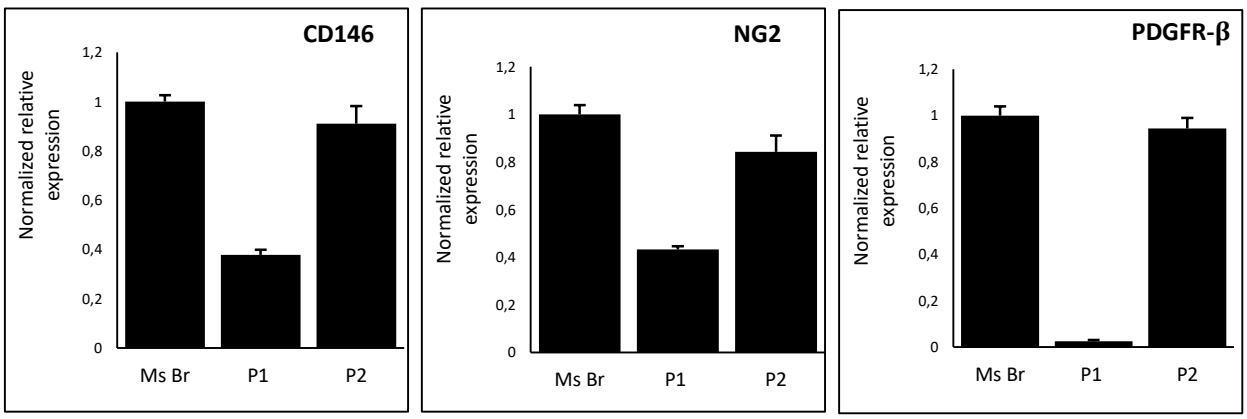

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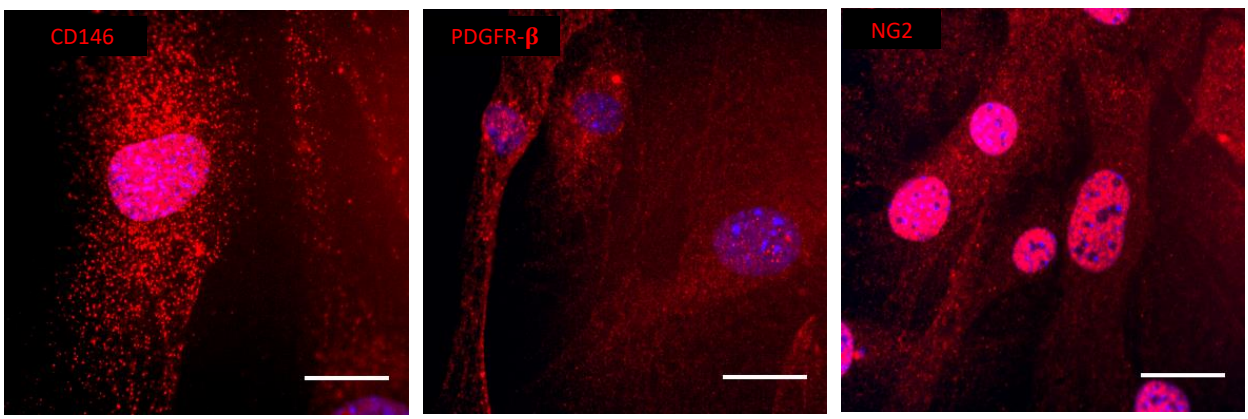

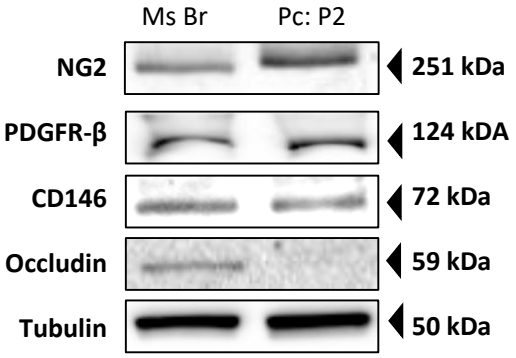

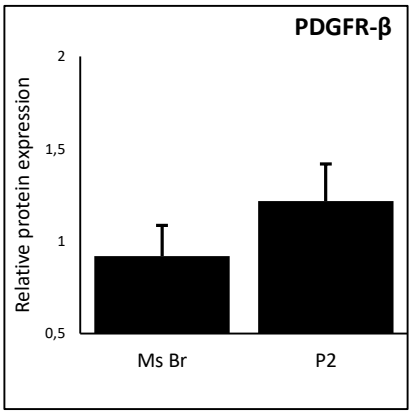

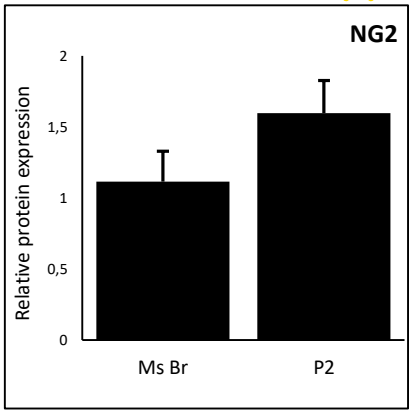

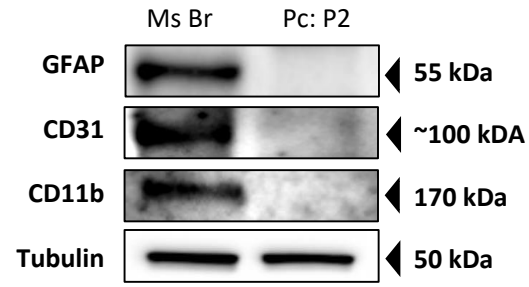

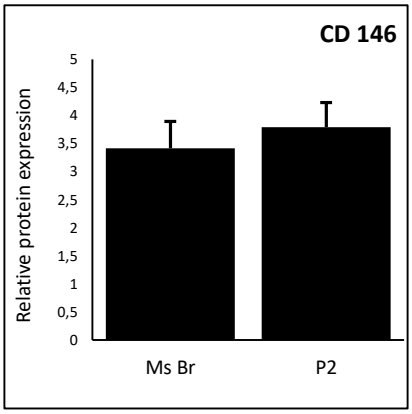

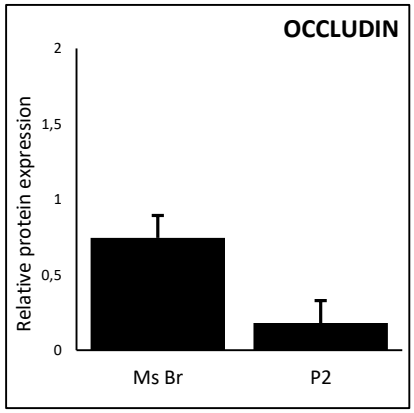

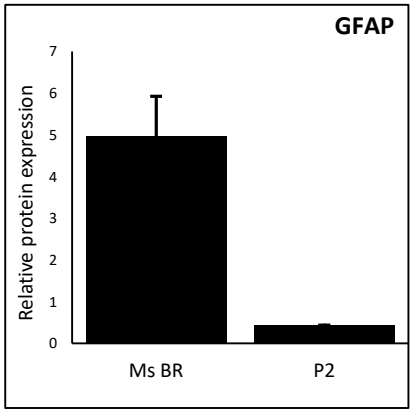

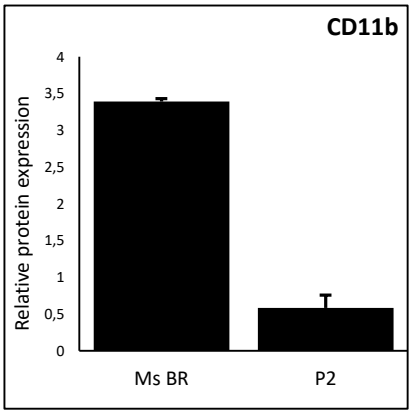

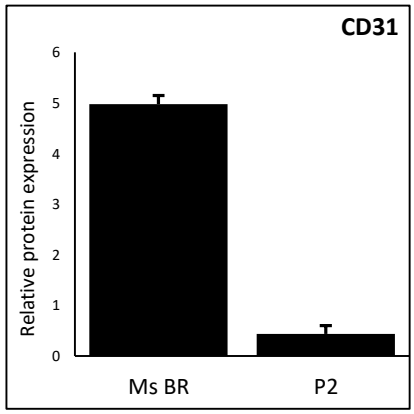

	Our protocol	Tigges *et al.*	Chen *et al.*	Crouch and Doetsch	Thomsen *et al.*	Yamazaki *et al.*
Treatment of tissue 	- meninges /white matter	No	- meninges /white matter	No	- meninges /white matter	- meninges /white matter
Tissue disintegration 	Dounce tissue grinder	21/18 gauge needle	Razor blade	Scalpel	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**
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/cm2. Cell culture dishes should be coated at least for 1 hour at room temperature.
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 KH2PO4, 2.86 g/L NaHPO4 (12 H2O), 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.
Hepes	Sigma	H-0887	Store at 4°C.
HBSS	Sigma	H-8264	Store at 4°C.
BSA	Sigma	A-8412	Store at 4°C.
dextran	Sigma	31398	
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.

ɔsyl 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.
DNase I	Sigma	11284932001	Prepare a 1000X stock solution by dissolving 100 mg in 10 ml sterile water and store at -20°C.
Glutamine	Merck	I.00289	Store at -20°C.
Gentamycin	Biochrom AG	A-2712	Store at 4°C.
Vitamins	Sigma	B-6891	Store at -20°C.
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.
Pericyte Medium-mouse	Sciencell research laboratories	1231	Store at 4°C.

Equipment Requirements

Surgical dissection tools (all components to be heat sterilized)	Forceps, scissors, Bunsen burner, cotton swabs, gauge
Laboratory equipment	Swing bucket rotor centrifuge Water bath with agitator Laminar Flow Hood : BSL2
	Dounce Tissue Grinder With Glass Pestle

Glassware (all components to be heat sterilized)	Pestle I: 0.0035 - 0.0065 inches Pestle II: 0.0010 - 0.0030 inches Vacuum filter assembly with coarse porosity fritted glass filter support base
Filtration tools	Sefar, Nylon mesh, 60-micron porosity