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Isolation and Cultivation of Primary Brain Endothelial Cells from Adult Mice

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[Abstract] Brain endothelial cells are the major building block of the blood-brain barrier. To study the role of brain endothelial cells *in vitro*, the isolation of primary cells is of critical value. Here, we describe a protocol in which vessel fragments are isolated from adult mice. After density centrifugation and mild digestion of the fragments, outgrowing endothelial cells are selected by puromycin treatment and grown to confluence within one week.

Keywords: Primary culture, Blood-brain barrier, Tight junctions, CD31, Occludin, Claudin-5, ZO-1, VE-cadherin

[Background] The blood-brain barrier protects the brain from uncontrolled entry of cells and substances. This is mainly achieved by brain endothelial cells that form a barrier composed of tight and adherens junctions to restrict paracellular transport.

This protocol was developed to overcome the limited availability of mouse brain endothelial cell lines that maintain their key characteristics, *e.g.*, the expression of sufficient amounts of tight junction proteins such as occludin, ZO-1 or claudin-5 to induce a high transendothelial resistance.

In addition, the isolation of brain endothelial cells from genetically modified mice allows investigating of gene-specific functions *in vitro*.

Using this method, we previously complemented *in vivo* studies demonstrating the importance of NF-κB signaling in brain endothelial cells for maintaining normal blood-brain barrier function (Ridder *et al.*, 2015).

Materials and Reagents

A. Materials

1. Multiwell plate (cell culture grade) (Greiner Bio One International, catalog number: 662160)
2. Cellulose chromatography paper (sterilize at 180 °C) (Whatman, catalog number: 3030-931)
3. 50 ml centrifuge tubes (cell culture grade) (Greiner Bio One International, catalog number: 210261)
4. 10 ml disposable pipette (Greiner Bio One International, catalog number: 607160)

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5. Mice (C57BL/6, age 6 weeks up to 1 year from Charles River, Germany or the in-house breeding facility)
6. Ice

B. Reagents

	Reagents	Manufacturer	Brand	Catalog number	Preparation	Aliquots	Storage	Stock concentration	Working concentration	Dilution
1	Hydrochloric acid (HCl) 1 N (sterile filtered)	Carl Roth		K025.1	1 L		RT		0.05 N	1 ml HCl 1 N + 19 ml H_2O, sterile
2	Dulbecco's PBS (DPBS)	Biowest		L0615-500	500 ml		4 °C	1x	1x	undiluted
3	70% EtOH (denatured)	Th. Geyer		2270			RT			
4	Isoflurane	Baxter		KDG9623			RT			
5	Collagenase/dispase (sterile filtered)	Roche Diagnostics		11097113001	500 mg/5 ml H_2O, sterile	200 µl	-20 °C	100 mg/ml	1 mg/ml	100 µl collagenase/dispase in 10 ml medium
6	DNase I	Roche Diagnostics		11284932001	100 mg/10 ml H_2O, sterile	100 µl	-20 °C	10 mg/ml	4 µg/ml	40 µl DNase I in 10 ml medium
7	N_α-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK)	Sigma-Aldrich		90182	14.7 mg/10 ml H_2O, sterile	200 µl	-20 °C	1.47 mg/ml diluted to 14.7 µg/ml	0.147 µg/ml	100 µl TLCK in 10 ml medium
8	Puromycin	Sigma-Aldrich		P8833	2.5 mg/10 ml H_2O, sterile	500 µl	-20 °C	0.25 mg/ml	8 µg/ml	32 µl puromycin in 1 ml medium
9	Trypsin-EDTA 0.25%	Thermo Fisher Scientific	Gibco™	25200-056	100 ml		-20 °C	1x	1x	undiluted
10	4% paraformaldehyde	Merck Millipore		1040051000	4% paraformaldehyde in DPBS		-20 °C			
11	CD31	BD	BD Pharmingen	557355						1:500

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12	α-smooth muscle Actin (α-SMA)	Acris Antibodies		DM001-05						1:200
13	Iba1	Wako Pure Chemical Industries		019-19741						1:100
14	Glial Fibrillary Acidic Protein (GFAP)	EMD Millipore		AB5541						1:400
15	Zona occludens-1 (ZO-1)	Thermo Fisher Scientific	Invitrogen	40-2200						1:500
16	VE-Cadherin	Santa Cruz Biotechnology		sc-6458						1:500
17	Claudin-5 (Cldn-5)	Thermo Fisher Scientific	Invitrogen	34-1600						1:500
18	Occludin (Ocln)	Sigma-Aldrich		SAB3500301						1:500
19	Mouse collagen, type IV	Corning		354233	890 mg/ml 0.05 N HCl	100 µl	-80 °C	890 mg/ml	50 µg/ml	56 µl collagen IV + 944 µl 0.05 N HCl
20	Dextran MW 60,000-90,000	Alfa Aesar		J14495	1 kg		RT		18%	5.4 g dextran in 30 ml DPBS
21	Penicillin/streptomycin (100x)	Biochrom		A2212	100 ml	1 ml	-20 °C	100x	1x	100 µl pen/strep in 10 ml medium/dextran
22	L-glutamine	Thermo Fisher Scientific	Gibco™	25030024	100 ml	1 ml	-20 °C	200 mM (100x)	2 mM (1x)	100 µl L-glutamine in 10 ml medium
23	DMEM-F12 w/o glutamine	Thermo Fisher Scientific	Gibco™	21331020	500 ml		4 °C	1x	1x	undiluted
24	DMEM w/o glucose	Thermo Fisher Scientific	Gibco™	11966025	500 ml		4 °C	1x	1x	undiluted

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25	Plasma-derived bovine serum (PDS)	First Link		60-00-810	500 ml	10 ml	-20 °C	100%	20%	10 ml PDS in 50 ml medium
26	Antibiotic/antimycotic (100x)	Thermo Fisher Scientific	Gibco™	15240062	100 ml	1 ml	-20 °C	100x	1x	100 µl AA in 10 ml medium/dextran
27	Heparin-sodium	Ratiopharm		PZN 003029843	1 ampule		4 °C	5,000 I.E./ml	750 I.E./50 ml	150 µl heparin in 50 ml medium
28	Endothelial Cell Growth Supplement (ECGS)	Sigma-Aldrich		E2759	15 mg/5 ml DPBS	500 µl	-20 °C	3 mg/ml	30 µg/ml	500 µl ECGS in 50 ml medium
29	18% dextran solution (see Recipes)									
30	Working medium (see Recipes)									
31	Digestion medium (see Recipes)									
32	Full medium (see Recipes)									

Note: Mouse collagen, type IV: Defrost stock vial slowly on ice at 4 °C overnight. Vortex thoroughly. Aliquot and store at -80 °C. The collagen concentration varies from lot to lot. Therefore, the amount of HCl added has to be adjusted for every new lot.

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Equipment

1. Refrigerator (4 °C)
2. Shaker
3. Sterile beakers 100-150 ml (sterilize at 180 °C)
4. Laminar flow work bench
5. Dounce tissue grinder, 15 ml, autoclave (Sigma-Aldrich, catalog number: D9938)
6. Scalpel
7. Tweezers (sterilize at 180 °C)
8. Centrifuge (Hettich Lab Technology, model: UNIVERSAL 320 R),
9. Fixed-angle rotor (Hettich Lab Technology, catalog number: 1620A)
10. Big scissors (sterilize at 180 °C)
11. Small scissors (sterilize at 180 °C)
12. Pipette or vacuum pump
13. Water bath
14. Microwave oven

Procedure

A. Preparations (Day 1)–Coating of wells with collagen

1. Defrost one collagen aliquot for 2 wells of a 6-well plate or an according volume for other well sizes (see Table 1) slowly (2-3 h) on ice in a refrigerator (4 °C)
   If necessary, defrosting aliquots in the refrigerator without ice is possible.

Table 1. Volume adjustment according to well size

Plate type	6 well	12 well	24 well
Collagen/well needed	500 µl	250 µl	200 µl

2. Dilute collagen to 50 µg/ml with 0.05 N HCl.
   Note: 0.05 N HCl aliquots can be stored at -20 °C.
3. Vortex thoroughly. At least 10 sec until small bubbles form (turn the tube to ensure that the viscous collagen stock solution does not continuously stick to the bottom).
4. Coat wells evenly with collagen solution (volume see Table 1).
5. Put the plate on a shaker, 1 h at room temperature, 25 rpm.
6. Move the plate to 4 °C for storage (overnight possible).
   Note: It is also possible to coat the plates on the day of endothelial cell preparation.

B. Isolation (Day 2)

1. Preparations:

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a. Ice
b. Sterilization of instruments
c. Approx. 50 ml DPBS in a 150 ml sterile beaker on ice (for each sample).
d. Disinfectant (70% EtOH) in a sterile beaker on ice (approx. 50 ml in 150 ml sterile beaker)
e. Switch on laminar flow and prepare cellulose chromatography paper, tissue grinder, scalpel, tweezers and 50 ml centrifuge tubes (one for each sample).
f. Precool centrifuge to 4 °C.

2. Anesthetize mice according to your local animal regulations. We use an overdose of isoflurane, which leads to breathing arrest within one minute. Decapitate the mouse with a big scissor and dip the head in ethanol (on ice). Remove the brain swiftly (Figure 1A) and store it in DPBS on ice. Repeat for all brains.
   a. Cut off cerebellum and olfactory bulb.
   b. Remove meninges by rolling the brains on cellulose chromatography paper using blunt tweezers.
   c. Cut cerebrum in 2 to 4 pieces and put the pieces in 5 ml working medium (4 °C). Repeat for all brains.
   d. Transfer brains with 5 ml working medium (4 °C) into a tissue grinder (Figure 1E) and homogenize (30 strokes with pistil A, 25 strokes with pistil B, Figure 1F). Use a maximum of 10 brains in one tissue grinder.
   e. Transfer homogenate into a 50 ml centrifuge tube. Rinse tissue grinder with 5 ml working medium (4 °C) and add to the homogenate (10 ml altogether).
   f. Centrifuge homogenate at 1,350 *x g*, 5 min, 4 °C. Remove supernatant carefully using a pipette or vacuum pump.
   g. Resuspend the pellet in 15 ml dextran solution and vortex extensively (2 min). The result is a white, cloudy, homogenous suspension (Figure 1G).
   h. Centrifuge at 6,080 *x g*, 10 min, 4 °C. In the meantime, supplement digestion medium with 100 µl collagenase/dispase, 40 µl DNase I and 100 µl TLCK each per 10 ml digestion medium. Pre-warm digestion medium to 37 °C.
   i. After centrifugation, remove the fluffy myelin layer (top, black arrows in Figures 1H and 1I) and the dextran as completely as possible. Use a 10 ml disposable pipette. Remove the filter of the pipette first if necessary.
   j. Resuspend the pellet (white arrows in Figures 1H and 1I) in 10 ml digestion medium (37 °C).
   k. Digest the tissue for 1 h 15 min in a 37 °C water bath (shake from time to time for 2 to 3 sec–approx. every 15 min).
   l. Centrifuge cell suspension at 1,350 *x g*, 5 min, room temperature. In the meantime, get the pre-coated plate from the refrigerator, fill sterile DPBS (10 ml per sample) in a centrifuge tube and heat it to 37 °C. Optionally, supplement full medium with puromycin and pre-warm to 37 °C (see step B2q).

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m. Remove digestion medium.

n. Resuspend pellet in 10 ml warm DPBS.

o. Centrifuge at 1,350 *x g*, 5 min, room temperature. In the meantime, remove collagen from the coated wells and wash twice with DPBS. DPBS from the second wash is left in the wells until cells are ready for seeding.

p. Remove DPBS and resuspend the pellet in full medium. 2.5 ml full medium per well for a 6-well plate. Use 4-6 brains per culture plate.

q. Mix cell suspension carefully before seeding to ensure even distribution.

r. Add puromycin as indicated in Table 2. (Can be added directly to the full medium, see step B2l).

Table 2. Culture volume according to well size

Cell culture plate	**6 well**	**12 well**	**24 well**
Cell suspension/well	2.5 ml	1 ml	500 µl
Puromycin/well	80 µl	32 µl	16 µl

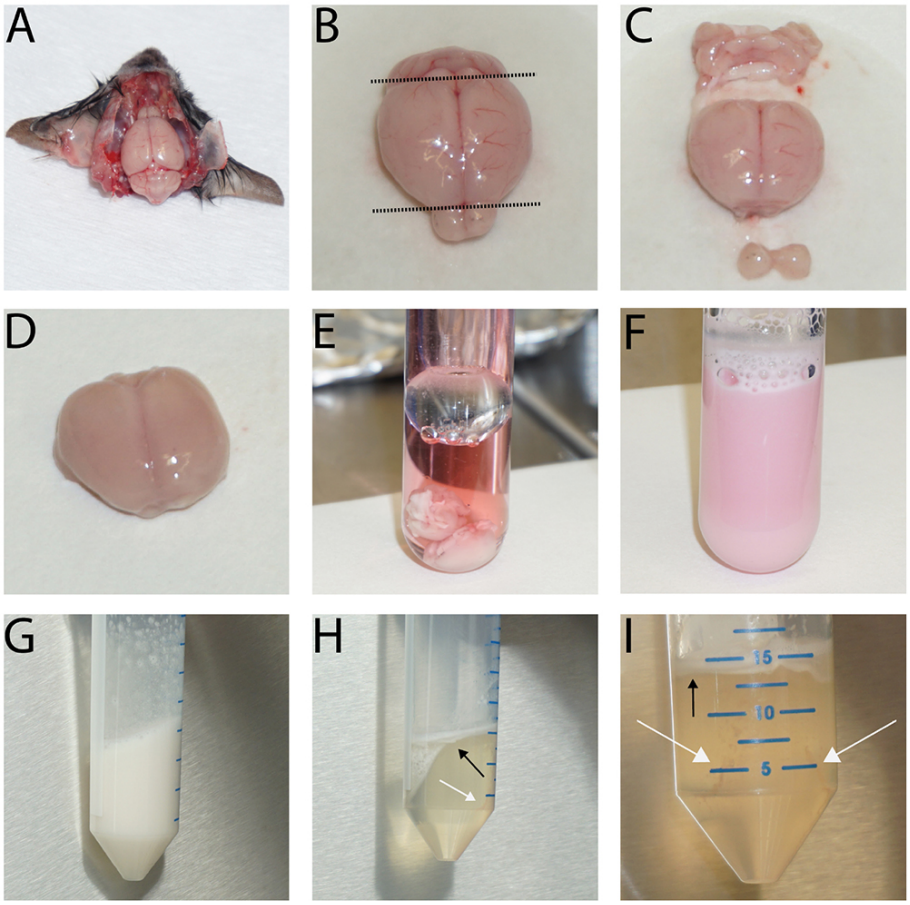

Figure 1. Typical images for the preparation of the brain, removal of meninges, homogenization and subsequent dextran gradient centrifugation. The images depict the first steps of the isolation procedure showing the brain *in situ* after removal of the skullcap (A), before (B. cut planes indicated by dashed line) and after removal of cerebellum, olfactory bulb

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(C) and the meninges (D). Then, collect the brains in a Dounce tissue grinder (E), homogenize them (F), and centrifuge the tissue homogenate. Next, resuspend the cells in the dextran solution and vortex extensively (G). Following the centrifugation, the resulting myelin layer is at the top while the vessel fragments collect around the edge of the tube bottom (H + I, black arrows: myelin layer, white arrows: pellet location). The size of the vessel fragment pellet depends on the number of brains used. In E-I, two brains were used for the preparation.

3. Day 3
   a. Wash cells twice with DPBS.
   b. Change full medium.
   c. Add puromycin (alternatively, puromycin can be added in advance to the full medium).
4. Day 4
   a. Change full medium.
      Note: No puromycin needed anymore.

C. Cultivation
1. Change medium 1-2 times per week, first time approx. 4-6 days after isolation.
2. Split the culture 1:2 (or 1:3) if the cells are confluent. Use trypsin 5-10 min and inactivate with full medium.
3. Plate cells and change medium the next day.

D. Purity of the cell culture (Figures 2 and 3)
Endothelial cells ($CD31^+$) > 95%
Pericytes (α-SMA^+) < 5%
No astrocytes ($GFAP^+$), microglia ($Iba1^+$), neurons ($NeuN^+$)

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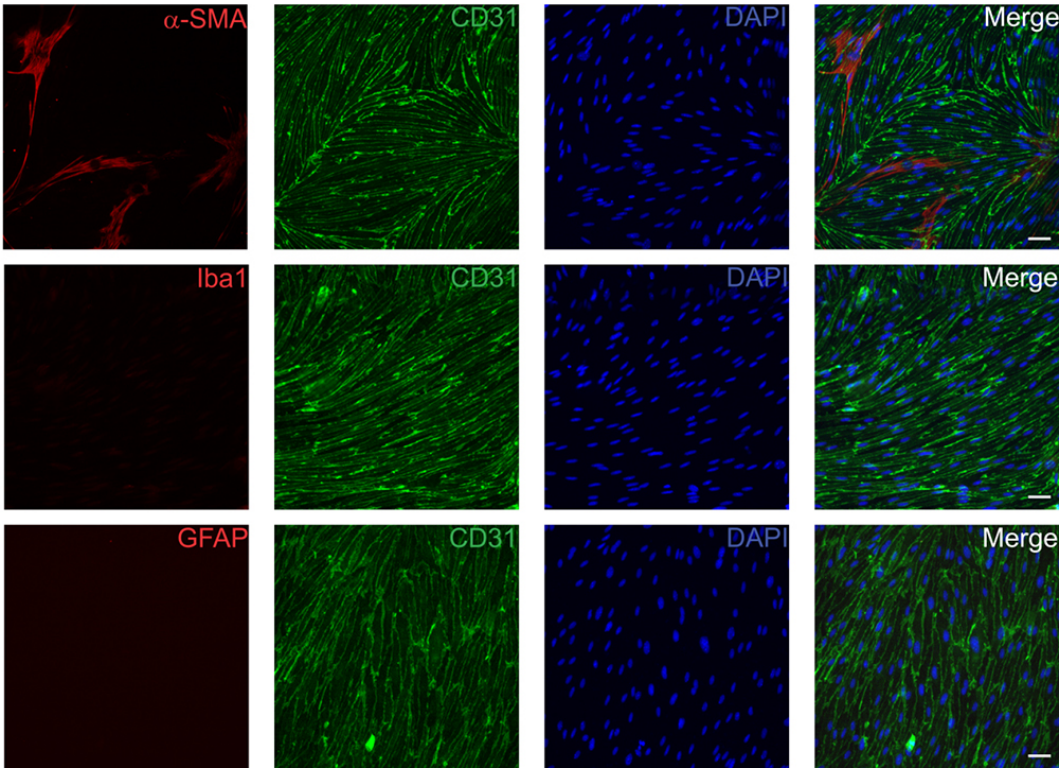

Figure 2. Representative immunofluorescence images of primary mouse brain endothelial cells. Cells were fixed with 4% paraformaldehyde 14 days after isolation and subsequently stained for CD31 (BD, 1:500) as an endothelial cell specific marker in combination with α-SMA (pericytes and smooth muscle cells, Acris, 1:200, upper row), Iba1 (microglia, Wako Pure Chemical Industries, 1:100, middle row) and GFAP (astrocytes, Millipore, 1:400, lower row). Scale bars represent 50 µm.

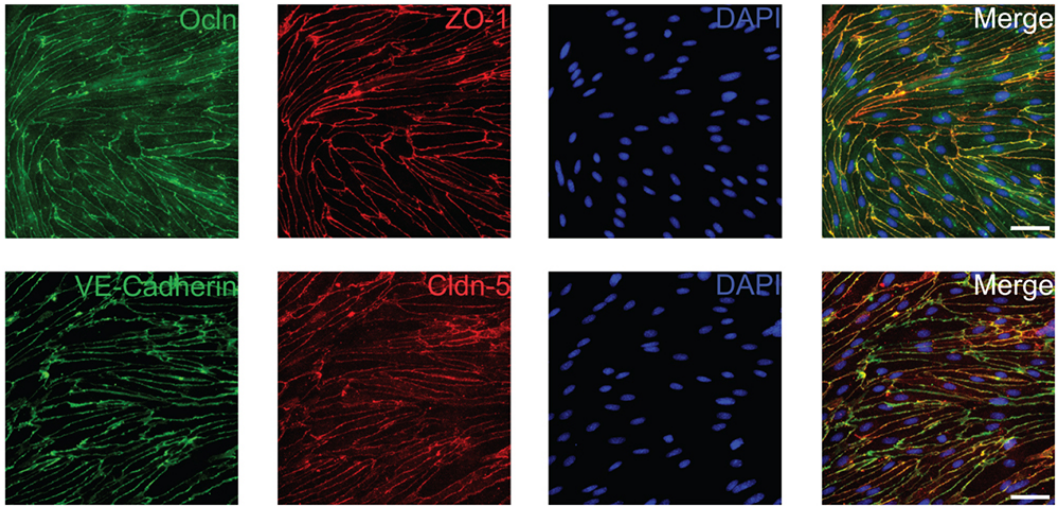

Figure 3. Primary mouse brain endothelial cells maintain expression of tight and adherens junction proteins. Cells were fixed 6-8 days after isolation with ice-cold methanol and subsequently stained for the tight junction proteins Ocln (Sigma-Aldrich, 1:500), ZO-1

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(Thermo Fisher Scientific, 1:500), Cldn-5 (Thermo Fisher Scientific, 1:500) and the adherens junction protein VE-Cadherin (Santa Cruz Biotechnology, 1:500). Scale bars represent 50 µm.

Data analysis

Primary mouse brain endothelial cells isolated by this method can be used for a variety of methods that include protein and gene expression analysis, assessment of transendothelial resistance using transwell inserts and transmigration or adhesion assays. In addition, these cells can also be grown on glass coverslips coated with collagen IV for live imaging, *e.g.*, to monitor intracellular calcium dynamics.

Notes

1. This protocol was developed to isolate brain endothelial cells from adult mice. We successfully isolated and cultured cells from young mice (6-8 weeks) as well as old mice (up to 1 year) in our laboratory without any modifications to the protocol. Using brains from other mouse strains has not been tested in our laboratory.
2. Cells usually reach confluence within 6-8 days (see Figure 4). They can be maintained in culture but are eventually overgrown by pericytes after several weeks.
3. After approximately 10 days cells do not adhere as strongly as before and are more likely to detach during staining procedures.

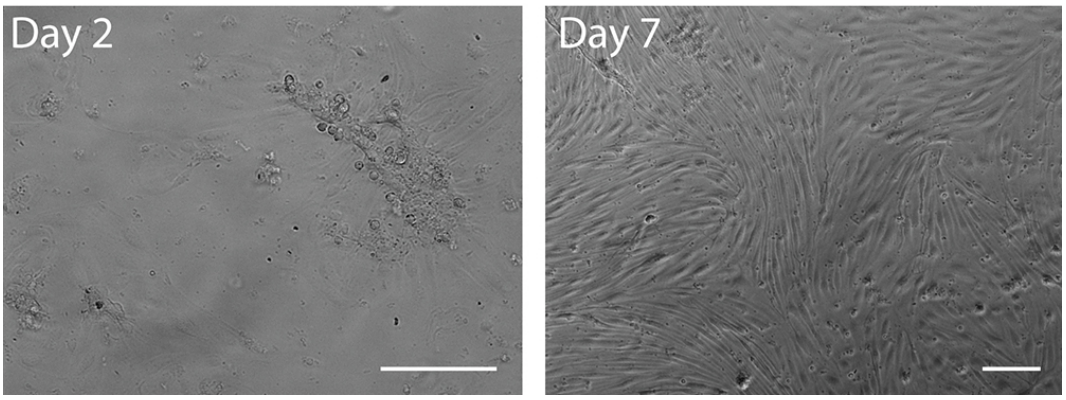

Figure 4. Bright field images of primary murine brain endothelial cells 2 days (left) or 7 days (right) after isolation. Note the attached vessel fragment and its radially outgrowing endothelial cells after 2 days in culture. Scale bars represent 100 µm.

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Recipes

1. 18% dextran solution (for 2 preparations)
   5.4 g dextran dissolved in 30 ml DPBS by heating (microwave)
   300 µl penicillin/streptomycin (100x)
   300 µl L-glutamine (200 mM)
   The solution can be stored at -20 °C and defrosted before usage, but penicillin/streptomycin and L-glutamine should be added after thawing
2. Working medium (for 2 preparations)
   20 ml DMEM-F12
   200 µl penicillin/streptomycin (100x)
   200 µl L-glutamine (200 mM)
3. Digestion medium (for 2 preparations)
   20 ml DMEM
   200 µl penicillin/streptomycin (100x)
   200 µl collagenase/dispase–add right before digestion
   200 µl TLCK–add right before digestion
   80 µl DNase I–add right before digestion
4. Full medium (max. storage time 3-4 weeks at 4 °C)
   40 ml DMEM-F12
   10 ml PDS
   500 µl antibiotic/antimycotic (100x)
   500 µl L-glutamine (200 mM)
   150 µl heparin (5,000 U/ml)
   500 µl ECGS

Acknowledgments

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