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TITLE:

Culture of Brain Capillary Pericytes for Cytosolic Calcium Measurements and Calcium Imaging Studies

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KEYWORDS:

Pericyte, ATP, Ca^{2+}-signaling, Fura-2 AM, Cal-520 AM, cell culture, plate reader, confocal laser scanning microscopy.

SUMMARY:

Brain capillary pericytes are essential players in the regulation of blood-brain barrier properties and blood flow. This protocol describes how brain capillary pericytes can be isolated, cultured, characterized with respect to cell type and applied for investigations of intracellular calcium signaling with fluorescent probes.

ABSTRACT:

Pericytes are associated with endothelial cells and astrocytic endfeet in a structure known as the neurovascular unit (NVU). Brain capillary pericyte function is not fully known. Pericytes have been suggested to be involved in capillary development, regulation of endothelial barrier tightness and trancytosis activity, regulation of capillary tone and to play crucial roles in certain brain pathologies.

Pericytes are challenging to investigate in the intact brain due to the difficulties in visualizing processes in the brain parenchyma, as well as the close proximity to the other cells of the NVU. The present protocol describes a method for isolation and culture of primary bovine brain capillary pericytes and their following usage in calcium imaging studies, where effects of agonists involved in brain signaling and pathologies can be investigated. Cortical capillary fragments are allowed to attach to the bottom of culture flasks and, after 6 days, endothelial cells and pericytes have grown out from the capillary fragments. The endothelial cells are removed by gentle trypsinization and pericytes are cultured for 5 additional days before passaging.

Isolated pericytes are seeded in 96-well culture plates and loaded with the calcium indicator dye (Fura-2 acetoxymethyl (AM)) to allow for measurements of intracellular calcium levels in a plate reader setup. Alternatively, pericytes are seeded on coverslips and mounted in cell chambers. Following loading with the calcium indicator (Cal-520 dye), calcium live-imaging can be performed using confocal microscopy at an excitation wavelength of 488 nm and emission wavelength of 510-520 nm.

The method described here has been used to obtain the first intracellular calcium measurements from primary brain capillary pericytes, demonstrating that pericytes are stimulated via ATP and are able to contract in vitro.

INTRODUCTION:

Brain capillary pericytes, together with endothelial cells and astrocytes, constitute the NVU[1,2,3]. The endothelial cells, which form the structural basis of the capillaries, form long cylindrical tubes with a diameter of 5-8 µm. The endothelial cells are sporadically covered with pericytes and surrounded by protrusions from astrocytes; the astrocyte endfeet.

The blood-brain barrier (BBB), situated at the brain capillaries, is the main site for exchange of nutrients, gases and waste products between the brain and the blood. The BBB also protects the brain from endogenous and exogenous neurotoxins and serves as a barrier for the delivery of a large number of drug compounds. The barrier function is a focus area, as well as an obstacle, for drug companies developing central nervous system (CNS) medicines. This has spurred a large interest in investigating the cells of the NVU in culture[4]. Brain astrocytes and endothelial cells have been cultured and characterized in a number of studies, whereas the studies and protocols for pericyte culture are sparse.

Previously published protocols have described generation of brain capillary pericyte cultures to some degree, using a range of different approaches such as immunopanning[5], high- and low-glucose media[6], fluorescent-activated cell sorting[7], density gradient centrifugation[8], etc. Although these methods seem sufficient to obtain cultures of pericytes, some are time consuming, cost expensive and the pericytes obtained might not be ideal due to the number of culture passages that can de-differentiate the pericytes[9]. Furthermore, the potential of cultured pericytes in in vitro signaling studies has been fairly unexplored until now.

The present work focuses on the generation of pericyte cultures from isolated bovine brain capillaries and the subsequent setup for measurements and imaging studies of changes in intracellular calcium, an important intracellular second messenger. We briefly describe the isolation of capillaries from cortical gray matter (for details see Helms et al.[10]) and the isolation and culture of pericytes in pure monoculture without contamination with endothelial or glial cells. We then provide a protocol for seeding of pericytes in 96-well plates and loading protocols for the calcium probe Fura-2 AM. Finally, we show how pericytes can be used in real-time confocal imaging in microscope culture chambers and describe the protocols for this.

PROTOCOL:

1. Preparation of buffers and solutions for cell culturing

1.1. Prepare collagen stock solution by dissolving 5 mg of collagen IV from human placenta in 50 mL of PBS overnight at 4 °C. Aliquot the stock solution into 5 mL portions and store at -20 °C.

1.2. Prepare fibronectin stock solution by dissolving 5 mg of fibronectin in 5 mL of sterile water overnight. Store the fibronectin stocks in aliquots of 500 µL at -20 °C. When thawing, add PBS to a final volume of 50 mL to prepare the work solution and store it at 4 °C.

1.3. Prepare Dulbecco's Modified Eagle Medium (DMEM) complete medium by adding 50 mL of fetal bovine serum (FBS), 5 mL of MEM nonessential amino acids and 5 mL of penicillin/streptomycin (0.1 g/L streptomycin sulfate and 100,000 U/L penicillin G sodium) to 500 mL of DMEM.

1.4. Prepare 5 mg/mL heparin stock solution by dissolving heparin sodium salt in PBS and pass it through a 0.2 µm filter for sterilization. Store the stock solution at 4 °C.

1.5. Prepare growth medium (GM) immediately before use; mix 10 mL of DMEM-comp and 250 µL of heparin stock solution per T75-flask.

2. Isolation of capillaries from fresh bovine brain

NOTE: Bovine brain capillaries are isolated and cultured as previously described (Helms et al.[10]).

2.1. Collect brains from calves, no older than 12 months of age, from a slaughterhouse and bring directly to the lab on ice.

2.2. Remove the meninges and collect all gray matter from the brain using a scalpel. Identify the meninges as the film covering the brain and the gray matter by its gray color.

2.3. Use a 40 mL Dounce tissue grinder to homogenize the gray matter in Dulbecco's Modified Eagle Medium (DMEM). Fill the slim part of the tissue grinder 1/5 with gray matter suspension and add DMEM until the slim part is filled.

2.4. Separate the capillaries from free cells and smaller tissue pieces by filtration of the homogenate through a 160 µm nylon net filter. Flush the filters with DMEM-comp. Retrieve the capillaries and pool the suspensions into 50 mL centrifugation tubes.

2.5. Resuspend the capillaries in DMEM-comp and add an enzyme mix of DNase I (170 U/mL), collagenase type III (200 U/mL) and trypsin (90 U/mL). Leave the suspension for 1 h in a 37 °C water bath for digestion of the capillaries.

2.6. Run the suspension through a 200 µm mesh filter and resuspend in FBS with 10% dimethyl sulfoxide (DMSO). Freeze the capillaries overnight at -80 °C and move them to liquid nitrogen the day after for long term storage.

NOTE: The protocol can be paused here.

3. Seeding and culturing of bovine capillaries

3.1. **Day 0:** Mix 0.7 mL of collagen IV stock with 6.3 mL of PBS. Add the solution to a T75-flask and leave the flask for 2 h at room temperature (RT) or leave it overnight at 4 °C.

3.2. Remove the collagen solution from the flask and wash three times with PBS.

3.3. Add 7 mL of fibronectin work solution and leave the flask for 30 min at RT. Then, remove the fibronectin solution and seed the capillaries immediately after.

3.4. During the 30 min waiting time, thaw one vial of capillaries in a 37 °C water bath.

3.5. When the capillaries are thawed, transfer immediately to a centrifugation tube with 30 mL of DMEM-comp and centrifuge for 5 min at 500 x *g* and RT. Remove DMEM-comp from the tube and re-suspend the capillary-pellet in 10 mL of fresh DMEM-comp.

3.6. Transfer the 10 mL suspension to the coated T75-flask and leave the capillaries to adhere to the bottom of the flask for 4-6 h in a 37 °C incubator at 10% CO_2.

NOTE: The cell growth rate is higher at 10% CO_2 rather than the conventional 5% CO_2.

3.7. After 4-6 h of incubation inspect the flask under a light microscope. Fractions of capillaries should now be attached to the bottom of the flask (**Figure 1**, day 0).

3.8. Prepare GM and aspirate the DMEN-comp medium very careful from the capillaries and replace it with 10 mL of freshly made GM.

3.9. **Day 2:** Remove GM from the capillaries and replace with 10 mL of freshly made GM. Cellular outgrowth from the capillaries should be visible under a light microscope at this point (**Figure 1**, day 2-3).

4. Isolation of primary pericytes from bovine brain capillaries

4.1. **Day 4:** Inspect the capillaries under a light microscope.

NOTE: The flask should now be approximately 60-70% confluent to provide an appropriate amount of pericytes (**Figure 1**, day 4). If this is not the case; replace the GM with 10 mL of fresh medium and leave the flask in the incubator for another day.

4.2. Aspirate the medium and wash the cells gently in PBS.

4.3. Add 2 mL of thawed Trypsin-EDTA for endothelial cells and leave the flask in the incubator for 1-3 min. Take out the flask frequently and observe with the microscope during this time period.

NOTE: The endothelial cells should round up and detach from the flask; pericytes should be visible as cells with a "ghost"-morphology and still be attached to the surface of the flask. **This is a tricky and important step.** It is essential to remove most endothelial cells to avoid contamination of the pericyte monoculture, but prolonged trypsinization can also detach the pericytes. The trypsinization time can vary slightly from time to time, and it is therefore of outmost importance to observe the flask frequently with the microscope during the treatment.

4.4. Gently tap the flask, when the endothelial cells have started to round up, to detach the loosened endothelial cells.

4.5. To stop the trypsinization, add 10 mL of DMEM-comp to the flask. Flush the flask carefully a few times with the medium to remove the endothelial cells. Aspirate the endothelial cell suspension from the flask. The endothelial cells can now be used for other purposes.

4.6. Add 10 mL of DMEM-comp to the flask. Look under the light microscope to assure the pericytes are still present and attached to the bottom. Put the flask back into the incubator to allow the pericyte-enriched culture to grow.

NOTE: It is important to observe the culture during the following days. If there is still a fair amount of endothelial cells growing another trypsin-treatment can be performed.

4.7. Allow the pericyte monoculture to grow with change of DMEM-comp. medium every second day. Check the growth of the cells under the light microscope (**Figure 1**, day 5-8).

5. Generation and storage of a monoculture of primary bovine pericytes

5.1. **Day 8-9:** Inspect the capillaries under a light microscope

NOTE: The pericytes should now have reached 70-80% confluency and grow in islands in the flask (**Figure 1**, day 9). If the confluency of the pericytes is less than 70%, allow the cells to grow for another day. The pericytes will not form a complete monolayer as the endothelial cells would.

5.2. Aspirate DMEM-comp and wash the pericytes with 7 mL of PBS.

5.3. Add 2 mL of trypsin-EDTA to the flask and leave it in the incubator for 2-3 min. Place the flask frequently under the light microscope to observe when the pericytes round up and detach

from the flask. When the pericytes have started to round up, the flask can be gently tapped to detach the cells.

5.4. Gently tap the flask, when the pericytes have started to round up, to detach the cells.

5.5. Add 10 mL of DMEM-comp to the flask to stop the trypsinization process. Flush the flask a few times with the medium to help detach the last pericytes.

5.6. Transfer the 12 mL cell suspension to a 50 mL centrifugation tube and fill up to 30 mL with DMEM-comp.

5.7. Centrifuge the cell suspension for 5 min at 500 x *g* and RT. Aspirate the DMEM-comp. carefully without touching the cell pellet. Resuspend the cell pellet in 3 mL of FBS with 10% DMSO.

5.8. Transfer the cell suspension into cryovials; add 1 mL to each, so there will be a total of 3 vials per T75-flask of pericytes. Freeze the pericytes at -80 °C overnight and move them to liquid nitrogen the day after for long term storage.

NOTE: Cells may be counted before freezing for a later estimate of survival percentage. The protocol can be paused here.

6. Setting up a pericyte monoculture for experiments

6.1. Coat a T75-flask with collagen IV and fibronectin using the same procedure as mentioned in section 3.1-3.4.

6.2. While the flask is being coated with fibronectin, thaw one vial of pericytes in a 37 °C water bath.

6.3. Transfer the now thawed pericytes from the cryovial to a centrifugation tube with 30 mL of DMEM-comp. Centrifuge the cell suspension for 5 min at 500 x *g*, RT.

6.4. Carefully aspirate the medium, leaving the cell pellet at the bottom of the tube. Re-suspend the pellet in 10 mL DMEM-comp.

6.5. Collect and transfer the cell suspension to the coated flask. Leave the flask with pericytes to grow in a 37 °C incubator at 10% CO_2.

6.6. Every second day, refresh the medium with 10 mL of fresh DMEM-comp.

NOTE: After 5 days of growth, the pericytes should have reached approximately 80% confluency. If the confluency is less, leave the cells to grow for another day or two. The cells should now be ready for seeding for further experiments.

7. Seeding of pericytes in a coated 96-well plate

7.1. Dilute collagen IV as described in step 3.1. Add 100 µL to each well in a 96-well plate and incubate for 2 h at RT or overnight at 4 °C.

7.2. Aspirate the solution and wash the wells three times with PBS.

7.3. Add 100 µL of diluted fibronectin to each well and incubate at RT for 30 min. Remove the fibronectin solution and use the plate immediately.

NOTE: Depending on how well the pericyte batch is growing, there should be enough cells for seeding two plates.

7.4. Take out the pericytes from the incubator and aspirate the medium. Wash the cells with PBS.

7.5. Add 2 mL of trypsin-EDTA to the pericytes and follow same procedure as in step 5.3-5.6.

7.6. Aspirate the medium, without harming the cell pellet and re-suspend the pellet in 1 mL of fresh DMEM-comp.

7.7. Take out 12 µL of cell suspension and add to a counting chamber. Under the light microscope, count at least 3 of 3x3 grids and use the average cell count per grid.

7.8. Use the equation below to calculate the volume of cell suspension that should be added to each well to seed 10.000 cells per well, in the 96-well plate.

$$\frac{cells}{mL} = count \times 10^4 \Leftrightarrow count = \frac{cell}{\frac{mL}{10^4}}$$

$$V_{per\ well} = \frac{10.000\ cells}{cells/mL} = \frac{10.000\ mL}{count \times 10^4} = \frac{1\ mL}{count} = X\ mL$$

7.9. Add DMEM-comp and the calculated volume of cell suspension in each well to reach a final volume of 200 µL.

7.10. Place the 96-well plate in a 37 °C incubator at 10% CO_2. Leave the cells to grow for 4 days with a change in media every second day.

8. Preparation of buffers and solutions for Ca^{2+}-imaging

8.1. Autoclave coverslip cell chambers and coverslips.

8.2. Assay buffer: Add 1.19 g of HEPES to 500 mL of HBSS buffer for a final concentration of

10 mM HEPES. Adjust the pH to 7.4.

8.3. Prepare 20% (w/v) Pluronic F127 + 1% (v/v) polyethoxylated castor oil stock solution by dissolving 0.5 g of Pluronic F127 solution in 2.5 mL of anhydrous DMSO in a glass vial. Heat to 40 °C for approximately 30 min or until dissolved and vortex. Add 25 µL of polyethoxylated castor oil and store at RT. Do not freeze.

8.4. Prepare 2 mM Fura-2 AM stock by dissolving 1 mg in 500 µL of anhydrous DMSO. Store in aliquots of 20 µL at -20°C protected from light.

8.5. Prepare 5 µM Fura-2 AM loading solution by mixing 20 µL of 20% Pluronic F-127 + 1% polyethoxylated castor oil stock solution with the 20 µL of 2 mM Fura-2 AM aliquot. Add 500 µL of assay buffer and vortex. Add assay buffer to a final volume of 8 mL. The solution should be prepared immediately before use and protected from light.

8.6. Prepare 4 mM Cal-520 AM by dissolving 1 mg in 226.7 µL of anhydrous DMSO. Store in aliquots of 20 µL at -20°C protected from light.

8.7. Prepare 20 µM Cal-520 AM loading solution by mixing 20 µL of 20% Pluronic F-127 + 1% polyethoxylated castor oil stock solution with the 20 µL 4 mM Cal-520 aliquot. Add 500 µL of assay buffer and vortex. Add assay buffer to a final volume of 4 mL. The solution should be prepared immediately before use and protected from light.

9. Loading of pericytes with Fura-2 AM calcium indicator dye in a plate-reader setup

NOTE: All solutions should be at RT before the experiment starts.

9.1. Take out the 96-well plate with cells from the incubator and aspirate the medium from the wells. Wash the cells twice with assay buffer.

9.2. Add 100 µL of loading solution to each well and wrap the plate with tinfoil, to avoid photo bleaching. Incubate for 45 min with 30 rpm shaking at RT.

NOTE: Do not load Fura-2 AM at 37 °C, as it may load internal compartments. Remember to leave wells with cells in assay buffer instead of loading buffer; this is the “blanks” used for measuring background fluorescence.

9.3. Aspirate the loading buffer and wash the cells with assay buffer twice. Add 100 µL of fresh assay buffer and leave the cells to incubate for 30 min at RT; this allows for continuous cleavage of the AM-ester and thereby trapping Fura-2 AM inside the cells.

9.4. Prior to the Ca^{2+}-imaging, wash and replace the buffer with 100 µL of fresh assay buffer.

10. Well-plate fluorescence reading of pericytes in a plate-reader setup

10.1. Set the temperature of the plate reader to 37 °C and transfer the 96-well plate with cells to the **sample plate** position. Place the reagent plate with agonist at the **reagent plate** position.

10.2. Start by measuring loading of the cells to ensure equal loading of Fura-2 AM in all wells.

10.3. Perform the measurements with excitation fluorescence wavelength at 340 nm/380 nm and the emission wavelength at 510 nm. Add 50 µL of agonist at speed 150 µL/s from the reagent plate to each well with cells in the **sample plate** position.

10.4. Save the data and export as xlsx files for further analysis. **Figure 2** shows the cytosolic Ca^{2+}-response measured as the ratio between the two excitation wavelengths over time, where background fluorescence is subtracted.

NOTE: The plate-reader need to be a dual microplate reader with room for a "cell tray" and a "sample tray" and an integrated pipettor system.

11. Seeding of pericytes in a coated cell chamber for live imaging

11.1. Mount a coverslip into the cell chamber and make it tight to avoid leakiness.

11.2. Dilute collagen IV as described in step 3.1. Add 500 µL to each cell chamber and incubate for 2 h at RT or overnight at 4 °C.

11.3. Aspirate the collagen solution and wash the chambers three times with 500 µL of PBS.

11.4. Add 500 µL of diluted fibronectin to each well and incubate at RT for 30 min. Remove the fibronectin solution and use the cell chamber straight afterwards.

11.5. In the meantime, take out the flask with confluent pericytes and wash with 7 mL of PBS.

11.6. Add 2 mL of trypsin-EDTA to the pericytes and follow same procedure as in step 5.3-5.6.

11.7. Proceed by following the same steps as in step 8.6-8.7.

11.8. Use the equation below to calculate the volume of cell suspension, which should be added to each chamber to seed 90.000 cells per chamber.

$$\frac{cells}{mL} = count \times 10^4 \Leftrightarrow count = \frac{cell}{\frac{mL}{10^4}}$$

$$V_{per\ chamber} = \frac{90.000\ cells}{cells/mL} = \frac{90.000\ mL}{count \times 10^4} = \frac{9\ mL}{count} = X\ mL$$

11.9. Add DMEM-comp and the calculated volume of cell suspension in each chamber to

reach a final volume of 500 µL.

11.10. Place the cell chambers in the incubator at 37 °C, 10% CO_2. Leave the cells to grow for 6 days (or until confluent).

NOTE: The pericytes grow slower on glass-slides compared to plastic; more days of growth are necessary.

12. Loading of pericytes with Cal-520 AM calcium indicator dye for live imaging

NOTE: All solutions should be at RT before the experiment starts.

12.1. Prepare the 20 µM Cal-520 AM loading buffer: Mix 20 µL of 20% Pluronic F-127 + 1% polyethoxylated castor oil stock solution with the 20 µL 4 mM Cal-520 aliquot. Add 500 of µL assay buffer and vortex. Add assay buffer to a final volume of 4 mL. The solution should be prepared immediately before use and protected from light.

NOTE: Protect solutions containing Cal-520 AM from light exposure.

12.2. Take out the cell chambers from the incubator and aspirate the medium. Wash the cells twice with assay buffer.

12.3. Add 500 µL of loading buffer to each chamber and incubate at RT for 45 min.

12.4. Aspirate the loading buffer and wash the cells twice with assay buffer.

12.5. Add 500 µL of fresh assay buffer to each chamber and incubate for 30 min at RT to allow cleavage of the AM-ester.

12.6. Replace the buffer with 500 µL of fresh assay buffer before performing the live imaging at a confocal microscope.

13. Live imaging of intracellular Ca^{2+}-levels

NOTE: A variety of microscope types can be used for the imaging. Upright or inverted conventional fluorescence microscopes, as well as upright or inverted confocal laser scanning microscopes with appropriate excitation source (488 nm) and emission filters (510-520 nm) can be used. Objectives should be suited for fluorescence and be of a high quality and with high numerical aperture (NA).

13.1. Mount the cell chamber on the stage of the confocal microscope as gentle as possible, in order to avoid disturbance of the cells.

13.2. Select an excitation wavelength of 488 nm, emission at 515 nm, sequential image

acquisition with 5 second intervals, an XY image size of 512 x 512 pixels and measure for 2 min to measure baseline calcium signals.

13.3. Add 3 µL of 100 mM ATP to the cell chamber with a pipette, and continue the sequential image acquisition. Perform the addition slowly and gently to not disturb the preparation and move the cells out of focus.

13.4. Observe the degree of changes and increase the time interval over time as needed for approximately 18 min until no further morphological change is noted (**Figure 3**).

13.5. Save time-lapse images and export them as TIFF and/or AVI files for further analysis.

NOTE: One vial of pericytes should give enough cells for seeding in 1-2 96-well plates and several coverslips, meaning you can prepare cells for both types of calcium-measurements.

REPRESENTATIVE RESULTS:

Bovine brain capillaries were isolated from fresh brain tissue and **Figure 1** presents the capillary seeding and cellular outgrowth over days and subsequent purifying of pericytes. The capillaries are fully attached to the flask at day 1 and on day 2 endothelial sprouting has become visible (**Figure 1**, day 2). After 4 days, the cellular outgrowth is highly distinctive (**Figure 1**, day 4a) and the endothelial cells are removed by gentle trypsinization as according to the described protocol. Remnants of the capillaries can be present after the trypsinization, but will disappear from the flask in the following days (**Figure 1**, day 4b-6). After removal of the endothelial-layer, pericytes are easily detected with morphology distinct from the endothelial cells. The pericytes present finger-like processes that attach strongly to the flask (**Figure 1**, day 4b). Subsequently, pericytes are allowed to grow until confluency (**Figure 1**, day 4b-9) and on day 9, the pericytes have reached approximately 80% confluency and grow in islands. This is in contrast to the endothelial cells that do create a contact inhibiting monolayer observed at day 4.

ATP is a well-known endogenous inducer of intracellular Ca^{2+}-signaling[11] and was used as an extracellular stimulant to induce cytosolic changes in Ca^{2+}-levels in pericytes. Addition of ATP to the Fura-2 loaded pericytes, as according to the described protocol, resulted in an increase in cytosolic Ca^{2+}-levels measured as the fluorescent ratio as shown in **Figure 2**. The ATP-induced response occurs immediately after addition of ATP to the pericytes and declines slowly over the measured time period.

Using this protocol for real-time confocal imaging of intracellular Ca^{2+}-responses, pericytes were seeded on coated coverslips, loaded with Cal-520 AM and placed at the confocal microscope. **Figure 3** (0 s) shows the pericytes with baseline levels of fluorescence prior to treatment. During live-recording, ATP is added to the pericytes and a strong intracellular Ca^{2+}-response is evident shortly after (64 s). Soon after, the cytosolic Ca^{2+} compartmentalizes in the cells and a reduction in cell area is visible (189 s). At 300 s. post start of the recordings, the cell area is heavily reduced and the Ca^{2+}-signal has declined to intensity close to the baseline fluorescence.

FIGURE AND TABLE LEGENDS:

Figure 1: Culturing of capillaries and isolation of pericytes. Capillaries have been isolated from fresh bovine brain and seeded in culture flasks on day 0. Outgrowth from the bovine brain capillaries and the following isolation of pericytes were followed over days with a light microscope. Day 4a shows the endothelial cell growth prior to treatment with trypsin to remove endothelial cells and day 4b shows the remnants immediately after the treatment. Day 8a shows the focus plane, where any capillary remnants would be visible, whereas day 8b are focused on the plane where the growth of the pericytes is visible. The arrows show the endothelial outgrowth from capillaries. The arrowheads point to single pericytes.

Figure 2: Representative example of intracellular calcium-measurement using Fura-2 calcium indicator dye. Primary pericytes have been seeded in 96 well plates and loaded with Fura-2 AM in order to visualize changes in intracellular calcium. 10 µM ATP is added to the pericytes at 30 s. and the cytosolic Ca^{2+}-response is measured as the ratio between the two excitation wavelengths; 340 nm and 380 nm over time. Scale bars are defined as standard deviation (N=3, n=1).

Figure 3: Representative example of intracellular calcium live-imaging using Cal-520 calcium indicator dye. Primary pericytes have been seeded in a cell chamber and loaded with Cal-520 in order to visualize changes in intracellular calcium and cell morphology. 600 µM ATP is added to the pericytes and snapshots from different time points during the live-imaging are presented here.

DISCUSSION:

In this study, we have presented a method to isolate primary pericytes from bovine brains. The described protocol allows culture of this otherwise rather inaccessible cell type. The subsequently obtained cell culture was a nearly homogenous population of pericytes, with only few or no contamination of endothelial and glial cells based on cell morphology and protein expression[12] . Furthermore, we demonstrated a simple and straightforward method to load the pericytes with calcium dyes for Ca^{2+}-imaging using two different methods, depending on the intended outcome.

One of the main issues when isolating primary cells from brain tissue is the limited access to tissue. In several earlier studies, rats and mice are the traditional model animals used, but the brain tissue from these small animals are sparse[6,13]. Isolation from human brain has also been conducted[14]; however, due to ethical issues this is not easily accessible. Hence, a high cell yield from an available source is preferred. The number of cell vials obtained from isolation from a single bovine brain outnumbers the amount obtained from either mouse or rat by far, making the bovine tissue advantageous compared to the other mentioned sources. Another advantage of the method described here is the low passage number to obtain a pure culture of pericytes. Passaging of mural cells can lead to de-differentiation[15] and therefore, should preferably be avoided. In this protocol, gentle trypsinization to remove the endothelial cell layer is a single step used to isolate the pericytes and therefore also one of the most critical steps described in

this protocol. If the trypsinization is prolonged, the pericytes will start to detach from the flask together with the endothelial cells, leading to only a small yield. On the other hand, only allowing a very short trypsinization time can cause an impure culture of pericytes. It is therefore of utmost importance to observe the cells very frequently during the trypsinization step. One should be aware of the morphologically differences between pericytes and endothelial cells to be able to distinguish the two cell types during the trypsinization procedure. Although, this step might require some training, the method is less time consuming and inexpensive compared to other methods used to obtain pure pericyte cultures[7,8,14]. Furthermore, the obtained culture of pericytes here showed the typical "ghost"-like morphology with finger-like processes and expressed several specific markers such as α-SMA, PDGFR-β and Nestin (data not shown here)[12], which are well known markers for primary pericytes in culture[1].

Here, we also presented a simple method for Ca^{2+}-imaging using fluorescent calcium indicator dyes. Loading of the pericytes with the dyes (Fura-2 and Cal-520) should be performed at RT and not at 37 °C. Several studies have performed loading of AM esters at 37 °C[16,17], but loading at this temperature can lead to the esters entering intracellular compartments such as the endoplasmic reticulum. This is not preferable, as the Fura-2 AM can bind to free Ca^{2+} trapped in the internal stores and thereby result in a lower increase in the fluorescent measurements. Hence, it can cause false results. Although, we have not experienced any major difficulties with loading of the pericytes at RT, if one is experiencing problems anyhow, one might consider optimizing loading by sonication of the loading solution for 5 min in an ultrasound bath prior to loading of the cells.

With the described method, intracellular Ca^{2+}-signaling together with contraction of the pericytes is readily observed during live recordings, which can also be quantified for further analysis. The method has previously been used to make the first demonstration of in vitro Ca^{2+}-signaling and contraction of brain capillary pericytes[12] . In previous studies various methods have been used to measure and quantify cell contraction as a consequence of extracellular stimulus[18-20]. However, observing the preliminary intracellular response directly followed by contraction of the cells was not a possibility in these studies. In the current study it is possible to observe the intracellular signaling and the following contraction of the cells and both measurements can be quantified in the same experiment. Thus, it eliminates additional experiments, which are more time consuming as well, and shows the direct link between intracellular Ca^{2+}-signaling and the morphological changes.

In conclusion, this study represents a simple and effective method for isolating and culturing primary brain pericytes. In addition, we demonstrate an easy and reproducible method to study intracellular Ca^{2+}-signaling in cultured pericytes. The methods described here should provide other researchers in the field with strong tools to study the pericyte biology and intracellular signaling in pericytes in vitro.

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DISCLOSURES:

The authors declare no competing financial interests.

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Figure 1

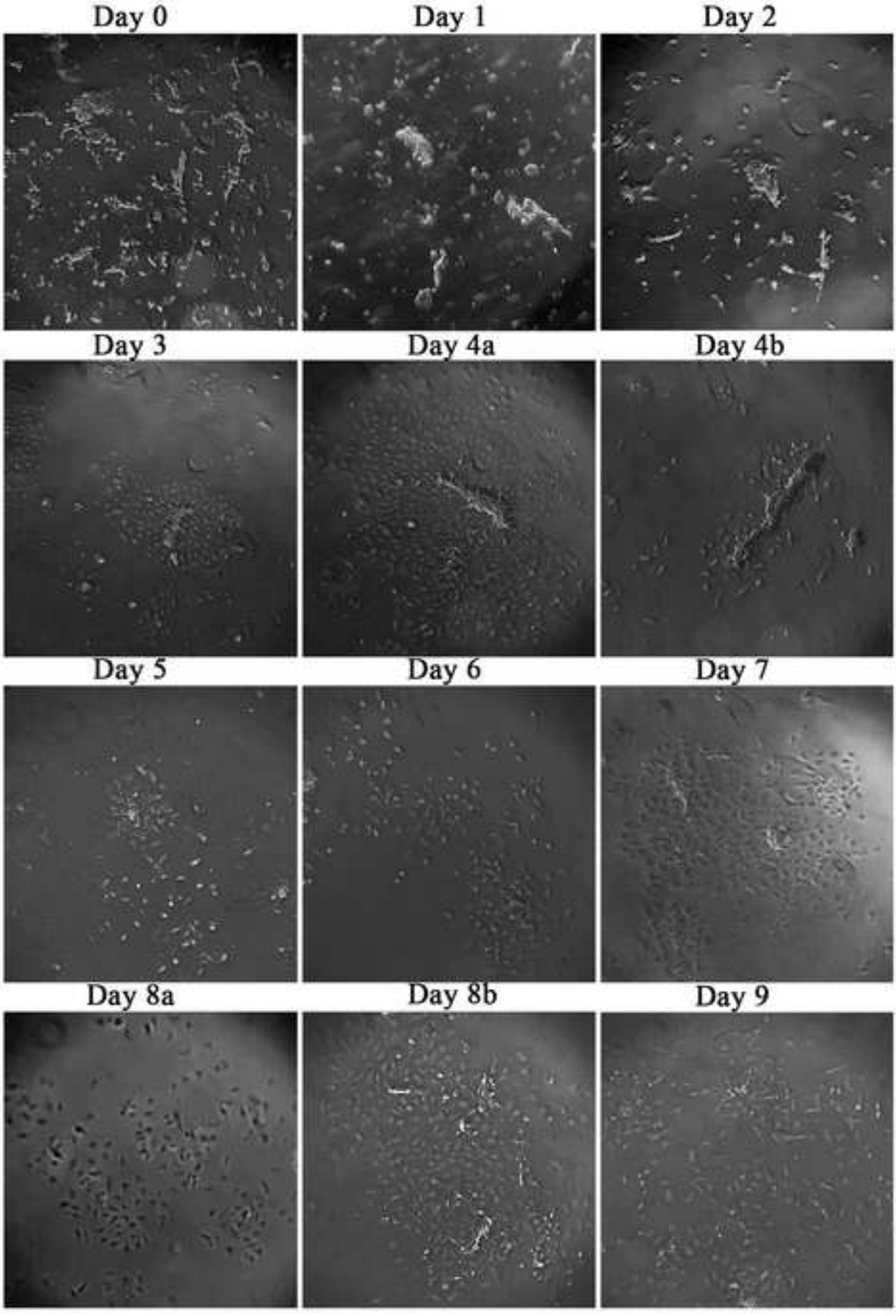


Figure 2

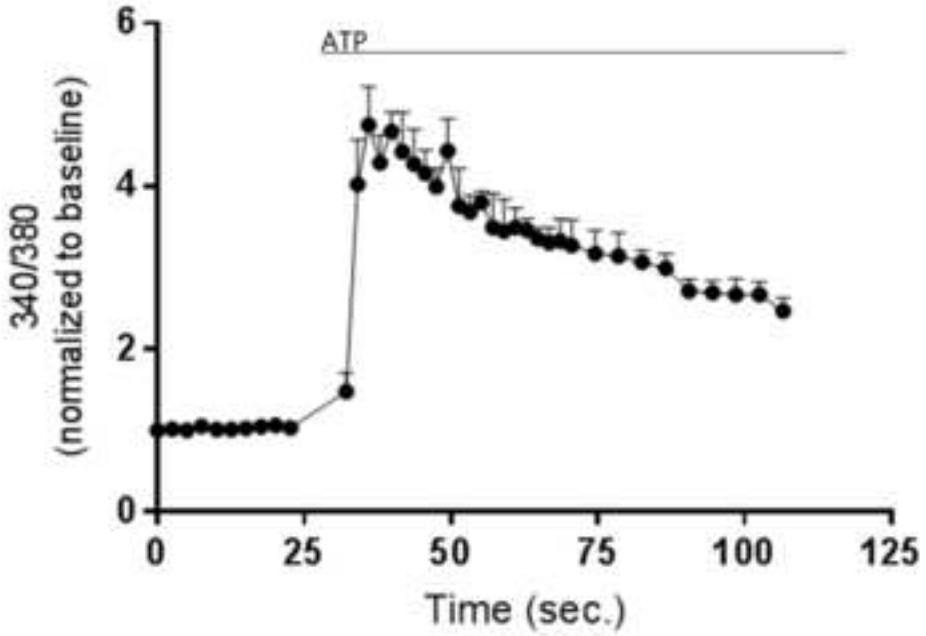
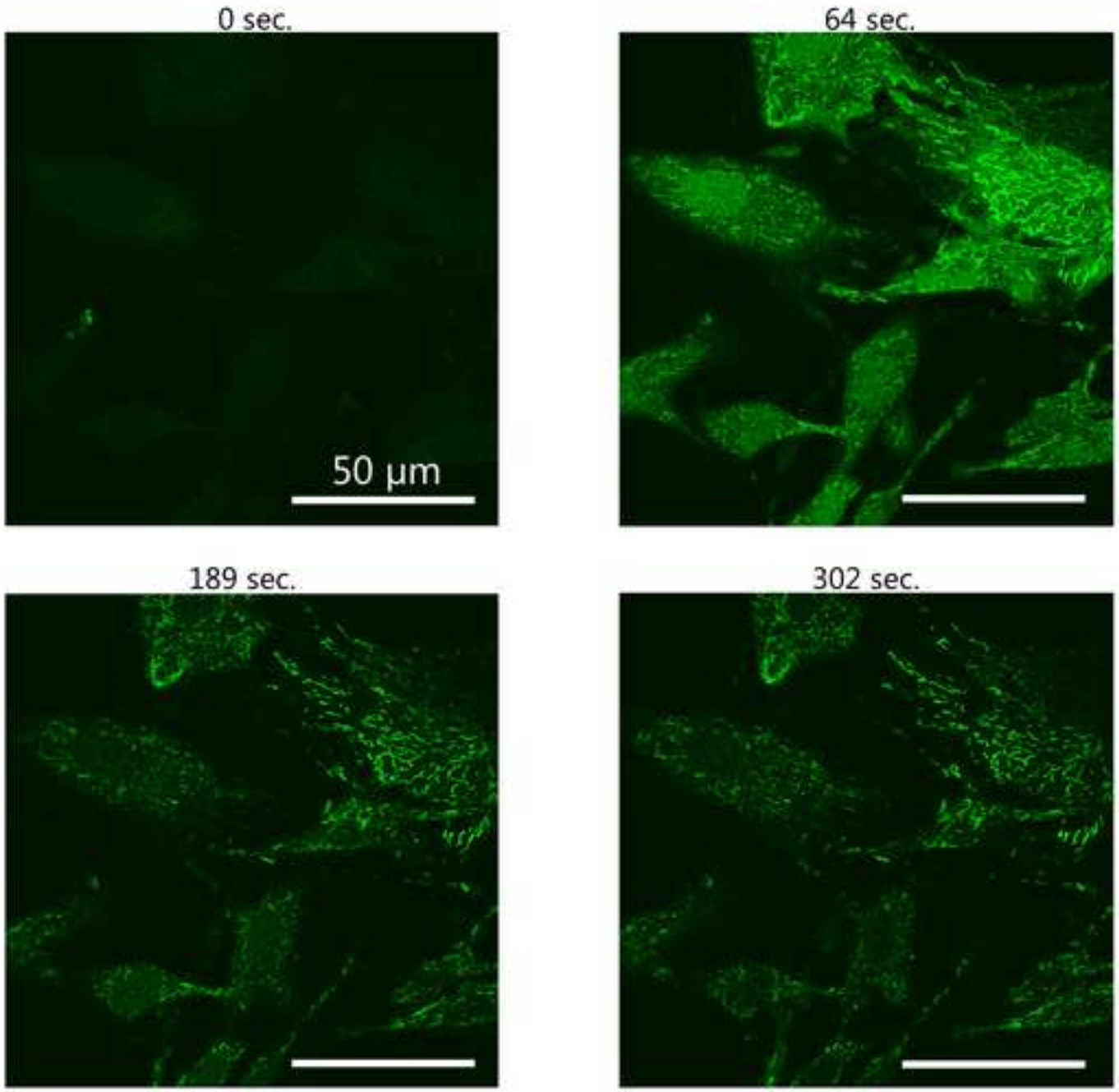


Figure 3



Name of Material/Equipment	Company	Catalog Number
ATP	Tocris	3245
Cal-520 AM	AAT Bioquest	21130
Cell incubator	Thermo Fisher	
Centrifuge	Thermo Fisher	Heraeus Multifuge 3SR+
Collagen IV	Sigma Aldrich	C5533
Confocal laser scanning microscope	Carl Zeiss	Zeiss LSM 510
Counting chamber	FastRead	102
Coverslip cell chamber	Airekacells	SC15022
Cremophor EL	Sigma Aldrich	C5135
DMSO	Sigma Aldrich	471267
Dulbecco's Modified Eagles Medium	Sigma Aldrich	D0819
Fetal bovine serum (FBS)	PAA/GE Healthcare	A15-101
Fibronectin	Sigma Aldrich	F1141
Fura-2 AM	Thermo Fisher	F1201
Glass coverslips 22x22 mm	VWR International	631-0123
HBSS	Gibco	14065-049
Heparin	Sigma Aldrich	H3149
HEPES	AppliChem Panreac	A1069
Light microscope	Olympus	Olympus CK2
MEM nonessential amino acids	Sigma Aldrich	M7145
Microplate Reader	BMG LabTech	NOVOstar
PBS	Sigma Aldrich	D8537
penicillin G sodium/streptomycin sulfate	Sigma Aldrich	P0781
Pluronic F127	Sigma Aldrich	P2443
Trypsin-EDTA	Sigma Aldrich	T4299
T-75 flask	Sigma Aldrich	CLS3972
96-well plate	Corning incorporated	3603

Comments/Description

Standard large volume centrifuge for spinning down cells

Inverted microscope

Formerly known as Kolliphor EL

Upright light microscope with phase contrast

Phosphate-buffered saline

We thank the editor and the reviewers for insightful comments and feedback. Below is listed a point-to-point rebuttal.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Authors response: The manuscript has been proofread

2. Please do not include citations in the abstract section.

Authors response: Citations has been removed from the abstract

3. Please define all abbreviation during the first time use.

Authors response: All abbreviation has been defines at first use

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

Authors response: For in-text formatting, corresponding reference numbers now appears as numbered superscripts after the appropriate statements.

5. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Authors response: The manuscript has been formatted as paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. A single space between each step, substep and note in the protocol has been included.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Authors response: All commercial language has been removed and replaced by generic temrs. All commercial products are referenced in the Table of Materials and Reagents.

7. Please ensure Fura -2 is not commercial.

Authors response: It has been ensured Fura-2 is not commercial

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Authors response: It has been ensured that all text in the protocol section is written in a imperative tense.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step. E.g., 4.3 etc.

Authors response: The Protocol has been so that individual steps contain only 2-3 actions per step.

10. Please ensure you answer the “how” question, i.e., how is the step performed?

Authors response: It has been ensure that it is explained how a step is performed

11. 1.3: How do you visually identify meninges and remove it? How do you identify the grey matter?

We have followed the reviewers suggestion and added the text "Note: The meninges areis identified as the film covering the brain and the grey matter is identified by its grey color."

12. 1.4: Volume used?

We have followed the reviewers suggestion and added the text "Fill the slim part of the tissue grinder 1/5 with gray matter suspension and add DMEM until the slim part is filled."

13. 2: Preparation of buffers and solution can be moved to a table format and uploaded separately as a .xlsx file to the editorial manager account.

Authors response: Preparation of buffers and solution has been changed to a table format and uploaded as an .xlsx file.

14. 7.8: We cannot film the calculation steps. Please remove the highlights.

Authors respond: The highlights have been removed

15. 8. This can be uploaded as a separate table.

Authors response: Preparation of buffers and solution for calcium imaging has been changed to a table format and uploaded as an .xlsx file.

16. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Authors response: The filmable content has been limited and should now comprise 2.75 pages or less.

17. As we are a methods journal, please ensure the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Authors response: It has been ensured that the Discussion explicitly cover the above mentioned in detail in 3-6 paragraphs.

18. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

Authors response: It has been ensured that the appearance of the refencese has been changed to JoVE Endnote style.

19. For all the images with microscope please include a scale bar and define it in the figure legend.

Authors response: It has been ensured that all images with a confocal microscope include a scale bar. Images with a light microscope does not include a scale bar, as the images sole purpose is to illustrate growth of the capillary- and cell culture.

20. Please sort the materials table in alphabetical order.

Authors response: The materials table has been sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors describe a straight forward protocol to generate pericyte cultures from isolated bovine brain capillaries and the subsequent setup for measurements and imaging studies of changes in intracellular calcium levels. Although protocols have been published to generate brain capillary pericyte cultures they are either time-consuming, cost expensive and cell numbers are not ideal to de-differentiate into pericytes during the culture. In this manuscript and subsequent video the authors describe in great detail the isolation of capillaries from cortical grey matter and the isolation and culture of pericytes in pure mono-culture without contamination. Subsequently they describe seeding of pericytes in 96-well plates and loading protocols for the calcium probe Fura-2 AM. This fluoro-probe can then be used for real-time confocal imaging in microscope culture chambers.

Minor Concerns:
The manuscript describes a clear cut protocol to generate pericyte cultures. The protocol is well written and the passages, which shall be included into the video sequences, are well selected. Although every concentration of every enzyme used is indicated, I would strongly suggest to include some more information (at least in the video) on how these enzymes should be handled. The problem with all these protocols arise when new investigators are trying to adopt such a protocol for the first time, not knowing that there are some issues with the usage of enzymes (e.g. batch number, activity differences between vendors or batches etc.). Therefore, I would like the authors to include a short section how to validate the enzymes for this assay, that new investigators can get a feeling how much enzyme for what time period to use.
Authors response: We have considered the reviewers suggestion and added the text "Note: The trypsinization time can vary slightly from time to time, and it is therefore of outmost importance to observe the flask frequently within the microscope during the treatment."

Reviewer #2:
Manuscript Summary:
This manuscript addresses a comprehensive protocol for the isolation and culture of brain capillary pericytes, and introduces two way of calcium measure for a broad spectrum of readers. The manuscript is interesting and the experiments have been carefully conducted. I think this version is suitable for the publish after correcting a few typos (as my quess).

Minor Concerns:
L131. It would be great if you can add an explanation for 10% CO2 condition instead 5% CO2 (conventional cond.).
Authors response: We have followed the reviewers suggestion and added the text "Note: The cell growth rate is higher at 10% CO2 rather than the conventional 5% CO2."
L254. "loading buffer" should be "assay buffer"
Authors response: We have followed the reviewers suggestion and changed the text to "assay buffer".
L353. 164s might be 190s (actually mentioned 189s, as in the figure 3)
Authors response: We have followed the reviewers suggestion and changed the text to "189 s."

Reviewer #3:

Manuscript Summary:
The manuscript by Sofie Hørlyck et al., describes a useful method to isolate pericytes from bovine brain. As the authors state study of pericytes neither in vitro nor in vivo is trivial. The method for isolation of pericytes presented here is similar to methods currently used to isolate these cells from other experimental animals with some modifications. The method is described in sufficient detail to be reproducible.
In addition, the authors present a method to detect changes in intracellular calcium - which is essential to study signalling in pericytes.

Minor Concerns:
An important issue in pericytes isolation is the purity of the culture. Therefore, a short characterization of the cultures (SMA, PDGFRbeta, or other pericyte marker) would further increase the usefulness of the manuscript.
Authors response: We have considered the reviewers suggestion. As mentioned in the discussion, the obtained culture of pericytes expressed several specific markers such as α-SMA, PDGFR-β and Nestin and the result is shown in a referenced study and thus, we believe the usefulness of the manuscript is ensured.

Reviewer #4:
Manuscript Summary:
The authors have submitted a manuscript entitled "Culture of brain capillary pericytes for cytosolic calcium measurements and calcium imaging studies" and described the protocols to cultivate and apply brain capillary pericytes for Ca+2 imaging.

The protocol is well written, I have only minor comments/suggestions for adaptions:

1.) Abstract

Line 35/36: Refs shouldn't be present in the abstract ?
Authors response: Citations has been removed from the abstract
Line 44: Alternatively to "before passage" use "before subcultivation" or "before passaging"
Authors response: We have followed the reviewers suggestion and changed the text to "before passaging"
Line 45: Alternatively to "Pericytes" use "Isolated pericytes"
Authors response: We have followed the reviewers suggestion and changed the text to "Isolated pericytes"
Line 49: Add also the emission wavelength
Authors response: We have followed the reviewers suggestion and added the text "and emission wavelength of 510-520nm."

2.) Introduction:

Line 63: barrier function is an obstacle for companies, more for the delivery of compounds ?
Authors response: We have followed the reviewers suggestion and added the text "…serves as a barrier for the delivery of…"
Line 69: point is missing after etc or write et cetera

Authors response: We have followed the reviewers suggestion and added a point.
Line 73: Alternatively to "up until now" use "up to now" or "until now"
Authors response: We have followed the reviewers suggestion and changed the text to "until now"
Lines 77-81: Authors used 3x "describe", should be replaced once (line 79) by e.g. "provide a protocol for"
Authors response: We have followed the reviewers suggestion and replaced (line 79) with "provide a protocol for"
3.) Protocol:

Line 98: explain the abbreviation DMEM-comp before the first/at the first appearance of this abbreviation
Authors response: the abbreviation DMEM-comp has been explained before the first appearance of this abbreviation
Line 107: Is collagen IV really dissolved in PBS and not in acetic acid ? I would assume it is dissolved in acetic acid, otherwise 3x PBS washing in the subsequent steps wouldn't be necessary. I was also wondering whether Coll IV is purified by chloroform extraction after dissolution.
Authors response: We have considered the reviewers pondering. However, the protocol description is correct and has not been changed.
Line 111: Disolving 5 mg fibronectin in 5 mL = 1 mg/mL, and then dilute 500 µL aliquotes to 50 mL volume would give a concentration of 0.01 mg/mL = 10 µg/mL, is this really the applied concentration and not rather low in comparison to other protcols (could be sufficient ?).
Authors response: We have considered the reviewers pondering. Even though the applied concentration might seem low, the protocol description is correct and has not been changed.
Line 126: Alternatively to "transfer" use "transfer immediately"
Authors response: We have followed the reviewers suggestion and changed the text to "transfer immediately"
Line 146: Alternatively to "in the microscope" use "with the microscope"
Authors response: We have followed the reviewers suggestion and changed the text to "with the microscope"
Lines 145-153: Maybe the authors could give an estimate, how long this detachment process could last in average.
Authors response: We have followed the reviewers suggestion and changed the in text 4.3 to "1-3 min."
Line 169: authors should state how often and with which volume they wash the pericytes
Authors response: We have followed the reviewers suggestion and changed the text to "7 mL PBS"
Line 182: add "T75" flask
Authors response: We have followed the reviewers suggestion and added the text "T75"
Line 203: maybe to comment on why pericytes are seeded on coll-IV/Fib and not e.g. poly-L-lysine as also done by several researchers.
Authors response: We have considered the reviewers suggestion and found the pericytes growth rate being high with the correct morphological appearance and marker expression when seeded on coll-IV/Fib and have not commented on other seeding options.
Line 257: Well-plate fluorescence reading is not really "imaging", the term "imaging" could be corrected
Authors response: We have followed the reviewers suggestion and changed the text to " Well-plate fluorescence reading"
Line 262: add the emission wavelength of your filter

Authors response: We have followed the reviewers suggestion and added the text "the emission wavelength at 510 nm"

Line 277: How often and with how much volume do you wash with PBS

Authors response: We have followed the reviewers suggestion and added the text "500 uL PBS" in 11.3 and "7 mL PBS" in 11.5.

4.) Discussion

Line 405: add space after PDGFR-b

Authors response: A space has been added after PDGFR-b

Line 427: is it really morphological "outcome"? is it more a link between Ca2+ and morphology per se or morphological structure ?

Authors response: We have followed the reviewers suggestion and changed the text to "morphological changes"

In summary, after the correction of these minor details, it could be recommended for acceptation.