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

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. Filming location: Will the filming need to take place in multiple locations? **No, different rooms same building**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Birger Brodin:** This is a simple and effective method for isolating and culturing primary brain pericytes to study intracellular Ca^{2+}-signaling.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Birger Brodin:** The obtained cell culture is a nearly homogenous population of pericytes and it is simple and straight forward to load the pericytes for Ca^{2+}-imaging.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Sofie Hørlyck:** 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.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Commented [AG1]: Authors: Since your shoot is fast approaching, I assigned the interview statements. If someone else ends up delivering each statement, please make a note of this change. Also, please memorize the statements that you are delivering for the day of the shoot.

Protocol

2. Isolation of Primary Pericytes from Bovine Brain Capillaries

2.1. Begin by thawing one vial of capillaries in a 37-degree Celsius water bath **[1]**. Once the capillaries have thawed, transfer them to a centrifuge tube with 30 milliliters of DMEM-comp **[2]** and centrifuge them for 5 minutes at 500 x *g* **[3]**.

2.1.1. WIDE: Establishing shot of talent placing a vial of capillaries in the water bath.

2.1.2. Talent transferring the capillaries to a centrifuge tube.

2.1.3. Talent putting the tube in the centrifuge and closing the lid.

2.2. Then, remove the medium from the tube **[1]** and re-suspend the pellet in 10 milliliters of fresh DMEM-comp **[2]**. Transfer the suspension to a coated T75-flask **[3]** and allow the capillaries to adhere to the bottom for 4 to 6 hours in a 37-degree Celsius incubator supplied with 10% carbon dioxide **[4]**.

2.2.1. Talent removing the medium from the tube.

2.2.2. Talent resuspending the pellet, with the medium container in the shot and labeled.

2.2.3. Talent transferring the capillaries to a T75 flask.

2.2.4. Talent putting the flask in the incubator and closing the door. *Videographer: Obtain multiple usable takes because this shot will be reused in 2.7.3.*

2.3. After the incubation, inspect the flask under a light microscope **[1]**. Fractions of capillaries should now be attached to the bottom of the flask **[2]**.

2.3.1. Talent using the microscope. *Videographer: Obtain multiple usable takes because this shot will be reused in 2.5.3 and 2.7.2.*

2.3.2. LAB MEDIA: Figure 1 Day 0 image only.

2.4. On day 4 after seeding the capillaries, inspect them under the microscope to ensure that they are approximately 60 to 70% confluent **[1]**. Aspirate the medium and gently wash the cells with PBS **[2]**. *Videographer: This step is important!*

2.4.1. LAB MEDIA: Figure 1 Day 4a image only.

2.4.2. Talent aspirating the medium and adding PBS to the cells, with the PBS container in the shot.

2.5. Add 2 milliliters **[0]** of thawed Trypsin-EDTA **[1]** and leave the flask in the incubator for 1 to 3 minutes **[2]**, taking the flask out frequently and observing cell detachment with the microscope **[3]**. When the endothelial cells start to round up, gently tap the flask to detach them **[4]**. *Videographer: This step is difficult and important!*

2.5.0 Added shot: Aspirate PBS

2.5.1. Talent adding trypsin to the flask, with the trypsin container in the shot.

2.5.2. Talent putting the flak sin the incubator.

~~2.5.3. *Use 2.3.1.*~~ NOTE: new shot, together with 2.5.4

2.5.4. Talent gently tapping the flask.

2.6. When the cells have detached, stop trypsinization by adding 10 milliliters of DMEM-comp to the flask **[1]**. Flush the flask a few times with the medium **[2]** and aspirate the endothelial cell suspension, which can now be used for other purposes **[3]**. *Videographer: This step is difficult and important!*

2.6.1. Talent adding medium to the flask. NOTE: This and next shot together *Videographer: Obtain multiple usable takes because this shot will be reused in 2.7.1.*

2.6.2. Talent flushing the flask.

2.6.3. Talent aspirating the endothelial cell suspension.

2.7. Add 10 milliliters of DMEM-comp to the flask **[1]** and check it under the light microscope to assure that the pericytes are still present and attached to the bottom **[2]**. Then, put the flask back into the incubator to allow the pericyte-enriched culture to grow **[3-TXT]**. *Videographer: This step is important!*

2.7.1. *Use 2.6.1.* NOTE: (I think) new shot

2.7.2. *Use 2.3.1.*

2.7.3. *Use 2.2.4.* **TEXT: Change medium every 2nd day**

3. Loading of Pericytes with Calcium Indicator Dye in a Plate Reader Setup

3.1. To seed pericytes into coated 96-well plates, detach them as described in the text manuscript **[1]**. Aspirate the medium, taking care to not disturb the cell pellet **[2]**, and resuspend the pellet in 1 milliliter of fresh DMEM-comp **[3]**.

3.1.1. Talent taking a tube with detached and spun down pericytes out of the centrifuge.

3.1.2. Talent aspirating the medium.

3.1.3. Talent resuspending the cells, with the medium container in the shot.

3.2. Count the cells using a counting chamber, then calculate the volume of suspension that should be added to each well to seed 10,000 cells per well **[1]**. Add the cell suspension **[2]**, then add enough DMEM-comp to the cells to achieve a total volume of 200 microliters per well **[3]**.

3.2.1. ~~Talent calculating the number of cells in the suspension.~~ NOTE: Authors will upload lab media

3.2.2. Talent adding cells to a few wells.

3.2.3. Talend adding DMEM to the cell suspension to achieve the correct volume per well.

3.3. When ready to load the pericytes with Fura-2 AM calcium indicator dye, take the 96-well plate with the cells out of the incubator **[1]** and aspirate the medium from the wells **[2]**. Wash the cells twice with assay buffer **[3]** and add 100 microliters of loading solution to each well **[4]**.

3.3.1. Talent taking the plate out of the incubator. *Videographer: After this step, the location is changed. The cell plate is taken to another lab, to perform the calcium studies.*

3.3.2. Talent aspirating the medium from a few wells.

3.3.3. Talent adding assay buffer to the wells and washing them.

3.3.4. Talent adding loading buffer to the wells.

3.4. Wrap the plate with tinfoil to avoid photo bleaching **[1]** and incubate it for 45 minutes with 30 rpm shaking at room temperature **[2]**. After the incubation, aspirate the loading buffer and wash the cells with the assay buffer twice **[3]**.

3.4.1. Talent wrapping the plate with tinfoil.

3.4.2. Talent putting the plate on a shaker and starting it.

3.4.3. Talent washing wells with assay buffer. NOTE: use 3.3.3.

3.5. Add 100 microlites of fresh assay buffer and leave the cells to incubate for 30 minutes **[1-TXT]**. After the incubation, set the temperature of the plate reader to 37 degrees Celsius **[2]** and transfer the 96-well plate with cells to the **sample plate** position **[3]**, then place the reagent plate with agonist at the **reagent plate** position **[4]**.

3.5.1. Talent adding assay buffer to the plate and leaving it to incubate. **TEXT: Replace buffer immediately before imaging**

3.5.2. SCREEN: Talent setting the temperature on the plate reader. *Videographer: Please film the screen for all SCREEN shots as a backup.*

3.5.3. Talent transferring the plate to the sample plate position. NOTE: This and next shot together

3.5.4. Talent placing the reagent plate with agonist at the reagent plate position.

3.6. Start by measuring loading of the cells to ensure equal loading of Fura-2 AM in all wells, then perform the measurements with excitation fluorescence wavelength at 340 to 380 nanometers and the emission wavelength at 510 nanometers **[1]**.

3.6.1. SCREEN: Talent measuring the loading of cells, then the fluorescence. NOTE: This and next shot together *Videographer: Please film the screen for all SCREEN shots as a backup.*

3.7. Add 50 microliters of agonist at a speed of 150 microliters per second from the reagent plate to each well with cells **[1]**.

3.7.1. SCREEN: Talent programming the reagent addition. *Videographer: Please film the screen for all SCREEN shots as a backup.*

4. Live Imaging of Intracellular Ca^{2+} Levels

4.1. Mount a coverslip into the cell chamber and tighten it to avoid leaks **[1]**. Add DMEM-comp and the calculated volume of cell suspension into each chamber for a final volume of 500 microliters **[2]**. Incubate the cell chambers at 37 degrees Celsius and 10% carbon dioxide for 6 days or until confluent **[3]**.

4.1.1. Talent mounting a coverslip into the cell chamber.

4.1.2. Talent adding medium and cell suspension into a chamber.

4.1.3. Talent placing the cell chambers in the incubator and closing the door. *Videographer: Once again, here the cell chambers are moved from the cell lab into another lab to perform the loading.*

4.2. Once the cells are confluent, take the cell chambers out of the incubator and aspirate the medium **[1]**. Wash the cells twice with assay buffer **[2]** and add 500 microliters of loading buffer to each chamber, then incubate them at room temperature for 45 minutes **[3]**.

4.2.1. Talent aspirating the medium from the cell chamber.

4.2.2. Talent adding assay buffer to the cells and washing them. *Videographer: Obtain multiple usable takes because this shot will be reused in 4.3.2.*

4.2.3. Talent adding loading buffer to a cell chamber and leaving it at room temperature. *Videographer: Obtain multiple usable takes because this shot will be reused in 4.3.3.*

4.3. After the incubation, aspirate the loading buffer **[1]** and wash the cells twice with assay buffer **[2]**, then add 500 microliters of fresh assay buffer to each chamber and incubate them for another 30 minutes at room temperature to allow cleavage of the AM-ester **[3]**.

4.3.1. Talent aspirating the loading buffer.

4.3.2. *Use 4.2.2.*

4.3.3. *Use 4.2.3.*

4.4. Replace the buffer with 500 microliters of fresh assay buffer and proceed with live imaging at a confocal microscope **[1]**. Mount the cell chamber on the stage of the microscope as gently as possible to avoid disturbance of the cells **[2]**.

4.4.1. Talent bringing the cell chamber to the microscope. *Videographer: This is also e new location (room in the same floor)*

4.4.2. Talent mounting the cell chamber.

4.5. Set the excitation wavelength at 488 nanometers, emission at 515 nanometers, 2-minute sequential image acquisition with 5 second intervals, and an XY image size of 512 by 512 pixels **[1]**. Add 3 microliters of 100 millimolar ATP to the cell chamber **[2]** and initiate image acquisition **[3]**.

4.5.1. SCREEN: Imaging parameters being set. *Videographer: Please film the screen for all SCREEN shots*

4.5.2. Talent adding ATP to the cell chamber.

4.5.3. SCREEN: Imaging being started. *Videographer: Please film the screen for all SCREEN shots*

Results

5. Results: Pericyte Isolation and Calcium Measurements

5.1. This protocol was used to purify pericytes form bovine brain capillaries. Cellular outgrowth from the seeded capillaries was imaged over the course of 9 days **[1]**. The capillaries were fully attached to the flask at day 1 and by day 2 endothelial sprouting was visible **[2]**.

5.1.1. LAB MEDIA: Figure 1.

5.1.2. LAB MEDIA: Figure 1, Day 1 and Day 2 images.

5.2. After 4 days, the cellular outgrowth was distinctive **[1]** and the endothelial cells were removed via trypsinization **[2]**. Remnants of the capillaries were present after the trypsinization but disappeared from the flask in the following days **[3]**.

5.2.1. LAB MEDIA: Figure 1, Day 4a image.

5.2.2. LAB MEDIA: Figure 1, Day 4b image.

5.2.3. LAB MEDIA: Figure 1, Day 4b – Day 6 images.

5.3. After removal of the endothelial layer, the pericytes were allowed to grow until confluency **[1]**. On day 9, the pericytes reached approximately 80% confluency and formed islands **[2]**.

5.3.1. LAB MEDIA: Figure 1, Day 5 – Day 8b images.

5.3.2. LAB MEDIA: Figure 1, Day 9 image.

5.4. Addition of ATP to the Fura-2 loaded pericytes resulted in an increase in cytosolic calcium levels **[1]**. The response occurred immediately after addition of ATP to the pericytes and declined slowly over the measured time period **[2]**.

5.4.1. LAB MEDIA: Figure 2.

5.4.2. LAB MEDIA: Figure 2. *Video Editor: Emphasize the jump in data after addition of ATP.*

5.5. The calcium response was also measured with real-time confocal imaging **[1]**. Baseline fluorescence was measured **[2]**, then ATP was added at 64 seconds and a strong intracellular calcium response was evident **[3]**.

5.5.1. LAB MEDIA: Figure 3.

5.5.2. LAB MEDIA: Figure 3 0s.

5.5.3. LAB MEDIA: Figure 3 64s.

5.6. Soon after, the cytosolic calcium compartmentalized in the cells and a reduction in cell area was visible **[1]**. By 300 seconds, the cell area was heavily reduced and the fluorescence declined almost to baseline levels **[2]**.

5.6.1. LAB MEDIA: Figure 3 189s.

5.6.2. LAB MEDIA: Figure 3 300s.

Conclusion

6. Conclusion Interview Statements

6.1. **Sofie Hørlyck:** This method 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.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.