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Perfusion Live Microscopy of VEC-GFP HUVECs Using LSM780/980 for Junction Morphology Analysis

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For live confocal microscopy with HUVEC VEC-GFP line

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Cell Culture

1 Thaw a HUVEC-GFP tube (stored in liquid nitrogen tank, should be in F5, F6 or F7) in the water bath.

NOTICE: As of 21.02.22, there are only 4 tubes of VEC-GFP HUVEC line remaining. If there are no more tubes ready, early passage HUVECs have to be transduced with VEC-GFP lentiviruses

2 In a 15 or 50 mL Falcon, mix the contents of the tube with 4 mL EGM2 media.

2.1 Centrifuge the Falcon for 4-5 mins @1000 RPM 5m

2.2 Suction the supernatant with a vacuum pipette, leave about 50 µL of supernatant. Flick the bottom of the tube 2-3 times or until observing a turbid smear covering the bottom

3 Add about 10 mL of EGM2 media that contains 10 µg/mL of Blasticidin S for maintaining cell selection(Lentivirus integration positive cells), transfer the mixture to a t75(Unless working with tubes with less than a million cells)

4 Change media every 2 days with EGM2+BSD

5 At 70-80% confluence, trypsinize the t75 in order to seed 2 6 well plates. For a minimum of 1.5 million cells following siRNA treatments, seed 4 wells for each condition. If planning to image for 2 conditions sequentially:

5.1 Seed first plate at 200k cells/well. This will be for the condition you will image first in 3 day time

6 Seed the second plate at 100-125k cells/well. This will be for the condition you will image second, in 4 days time

siRNA treatment

7 **For 4 wells in a 6 well plate:**

In a 1.5 mL eppi, mix 20 µL siRNA(10 µM) and 780 µL Lonza **EBM2** media **WITHOUT ANTIBIOTICS**
In a Falcon tube, mix 8 µL Dharmafect with 792 µL Lonza **EBM2** media **WITHOUT ANTIBIOTICS**

Incubate at RT for 5 minutes

8 Add the contents of eppi into the falcon tube, homogenize by gently pipetting up and down. Incubate in water bath for 20 minutes alongside a falcon aliquot of ***EGM2*** media **without antibiotics**.

9 Add 6.4 mL of prewarmed EGM2 media without antibiotics to the transfection mix

10 Remove old media from the first 6 well plate and add in 2 mL aliquots the incubating mixture

11 Repeat steps 7-10 for the second plate the next day

Seeding

12 Following 21-22 hours post siRNA treatment, gelatinize 2-3 0.4 Luer ibidi u-slides. Incubate at 37C until step 13

13 Following 24 hours post siRNA treatment, trypsinize the wells and seed cells to the uslides using EGM2(can be with antibiotic BSD) at a concentration of 2.25 million/mL. at a 100 µL volume per slide

13.1 20-30 minutes after seeding, add 120 µL EGM2

Live Cell Imaging

14 At a concentration of 1:1000, mix spy650 DNA dye with 500 µL of CO2 independent media
At a concentration of 1:2000, mix spy650 DNA dye with 7 mL of CO2 independent media

Following a 20 min incubation at 37C, change media in slide to image with the dye-media mix. Incubate for 2-3 hours

15 Calibrate Köhler illumination per LSM 780/980 instructions.

16 Set up the microscope flow units inside the microscope incubator. Through ZEN, set the incubators to 37C. Check that the incubator water bottle is sufficiently full. Start incubation

17 Start the equilibration program for ibidi perfusion system, do the pinch test(refer to ibidi flow manual if you never did this. HOWEVER, if you never did this, DO NOT DO IT FOR THE FIRST TIME INSIDE THE MICROSCOPE INCUBATOR)

Run the program for 30 minutes

18 In the meantime, set up the following imaging program:

Time acquisition: In 2 blocks(3 if you find the 5 hour pause function):
1) 1 min interval imaging for 30 minutes
2) 90 min acqusition pause for recovery
10 hour loop

Tile: 2x2, alternatively 4 seperate positions
Z-Level: 3-4 slices per view, make sure there is good vertical overlap
Resolution: Using 63x, 1024*1024 with 1.0 Zoom

19 Plug the slide to the flow units, making sure that the red coupler is connected to the right inlet

for left-to-right flow

20 Find Focus with Definite Focus and Store Focus following manual tweaking.

21 Do test acquisitions. Check GFP, T-PMT and SPY650 channel for any issues

Especially for GFP laser, aim to keep laser power as low as possible while maintaining good junction contrast

22 Start flow at 20 dynes/cm2. Do image acquisition tests again. Keep the perfusion running for 5 hours prior to imaging

23 Start imaging. Check the intensity decay/bleaching rate after 30 mins for future acquisition readjustments