Immunostaining of TRPV1 and its co-localization in mitochondria in rat VSMC

Materials:

12-well plate

Sterile precision coverslips

Sterile-filtered 0.1% gelatin dissolved in PBS (aliquoted in 20 ml aliquots in glass bottles, autoclaved and sterile-filtered before use)

MitoTracker® Deep Red FM (Ex/Em 644/665 nm), dissolved in 92 µl sterile DMSO to 1 mM aliquots, according to Invitrogen, this dye should endure the subsequent fixation and permeabilization

Vanilloid R1/TRPV1 Antibody (Novus Biologicals, against N-terminal end, polyclonal, rabbit, NBP1-97417)

Goat anti rabbit IgG (H+L), FITC labeled (Ex/Em 495/518 nm), Caltag Laboratories, L42001

Anti-VR1 antibody [BS397] – C-terminal (Abcam, ab203103)

Anti-Mouse IgG (whole molecule) – FITC antibody produced in goat (Sigma, F0257-.5ML)

10% goat serum in 1xTBS-T

1xTBS-T

4% paraformaldehyde (methanol-free) in growth medium or in PBS

0.2% Triton® X-100 in PBS

PBS

Growth medium

Mounting medium – Fluoromount™ Aqueous Mounting Medium, Sigma, F4680-25ML

Hoechst dye 34580 (Ex/Em 392/440 nm) or 33258 (Ex/Em 352/461 nm)

Nail polish

Dark chamber with parafilm and wet sponge

Confocal microscope (Leica Microsystems, Wetzlar, Germany)

Procedure:

Day 0

1. Put sterile precision-coverslips into the wells of a 12-well plate using sterile techniques and cover the whole well with 0.3 ml of 0.1% gelatin solution, and incubate at 37°C for 30 min

2. Aspirate the extra gelatin solution using a sterile Pasteur pipette

3. Seed 50 000 cells/well in a 12-well plate and let them grow o/n on gelatin-coated coverslips:

Samples in duplicates: a) unstained cells, b) only Hoechst stained cells, c) only MitoTracker DeepRed stained cells, d) only TRPV1 stained cells, e) only secondary antibody stained cells and f) growing cells stained with both TRPV1 and MitoTracker DeepRed and counterstained with Hoechst

Day 1

4. Change the fresh growth medium to cells and incubate them with pre-warmed growth medium supplemented with 250 nM MitoTracker® Deep Red FM for 30 min in the incubator (100-500 nM for 15-45 min recommended by the company)

(5. Replace the staining solution with prewarmed growth medium and observe cells using a fluorescence microscope or a fluorescence microplate reader)

From now on, the incubation steps should be performed in the dark:

6. Wash cells in pre-warmed PBS or growth medium

7. Incubate cells with 500 µl/well of 4% paraformaldehyde in growth medium for 10 min at 37°C

8. After fixation, rinse the cells several times in PBS

9. Incubate cells in 500 µl/well of 0.2% Triton® X-100 in PBS for 10 minutes

10. Wash 3x in 1xTBS-T (each washing step 5 min)

Following incubation steps are to be performed in dark, moist conditions, so the coverslips do not dry out:

11. Blocking in 10% goat serum (in 1x TBS-T) for 1 hour at RT by pipetting 20 µl of blocking solution on the parafilm and covering the drop with the coverslip facing cells inwards

12. Wash the coverslips 3x in 1x TBS-T

13. Incubate the coverslips on 20 µl droplets of TRPV1 primary antibody:

i) Novus Biologicals, N-terminal, polyclonal, rabbit, dilution 1:40 in 1xTBS-T for 2 hours at RT or o/n at 4°C

ii) Abcam, C-terminal, monoclonal, mouse, dilution 1:500in 1xTBS-T for 2 h at RT or o/n at 4°C

iii) For the sec. AB ctrl: only in 1xTBS-T

14. Wash the coverslips 3x in 1xTBS-T

15. Incubate coverslips on 20 µl droplets of FITC-labeled secondary antibody in 10% goat serum in 1xTBS-T (1:400 dilution for both mouse and rabbit) for 1 hour at RT

16. Wash the coverslips 3x in 1xTBS-T

(17. Counterstain the nucleus with 0.1-1 µg/ml Hoechst stain for 1 min and rinse with PBS)

18. Mount coverslips with a drop of mounting medium on the preparation glass

19. Seal coverslips with nail polish to prevent moving and drying the sample

20. Observe the samples under the confocal microscope (beware to use low laser power, because FITC does not have a high quantum yield, high laser power might cause samples to photobleach)

21. Use single stained samples for channel compensations, analyze samples by sequential scanning, make sure you do not see any green fluorescence in samples incubated with only secondary FITC antibody