Dihydroethidium (DHE) Staining for Superoxide in VSMC
DHE Excitation/Emission maxima=510/595 nm

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1. Grow cells to 70-80% confluency in 10% calf serum (CS).
2. Replace medium with 0.1% CS overnight (~ 12 hr).
3. Stimulate with the agonist (e.g., PDGF) for 4-6 hr in the dish (for long term stimulation) or, alternatively if one studies short term agonist-induced superoxide production, incubate with agonist just before adding dihydroethidium.
4. Trypsinize/detach cells from dish gently; quench the trypsin with 2% CS.
5. Centrifuge at 400 X g 5 min at room temperature (RT).
6. Aspirate the trypsin/2% CS/DMEM and wash once with 5 ml RT PBS-1X; resuspend the cells gently by pipetting up/down.
7. Resediment cells by repeating Step 5.
8. Resuspend cells in colorless HBSS at ~1-2 ml/10^6 cells.
9. Add 2 ml of cell suspension to the Falcon 2052 tubes (FACS tubes) and wrap them in aluminum foil.
10. Add DHE at final 10 μM (see protocol for DHE stock). (DHE IS VERY SENSITIVE TO LIGHT!!!)
11. Add the agonist for short term stimulation (Ang II, PDGF, etc.)
12. Incubate at 37 ° C for 30 min in the dark (can go up to 60 min if necessary) in the water bath.
13. Stop the reaction by placing the cells on ice.
14. Analyze by FACS analysis the fluorescence intensity of DHE.

To make DHE stock:

1. To make a 10 mM DHE stock, add 315 μl of DMSO in the 1 mg (pre-weighed vial) of DHE (Molecular Probes). Cover the vial in aluminum foil.
2. Vortex for 30 sec-1 min and make sure that the DHE is homogeneously resuspended and gives a pink color without clumps. Make working aliquots and keep them at -20 to -80 ° C.
3. Keep stocks on ice until the incubation with cells. Needs to be thawed at RT as DMSO freezes on ice.