

## **CM-H<sub>2</sub>DCFDA staining for H<sub>2</sub>O<sub>2</sub> in RASMs** (chloromethyl-2',7'-dichlorofluorescein diacetate--Ex/Em: 502/523 nm)

- References:
1. Royall, J. and H. Ischiropoulos. 1993. Arch. Biochem. Biophys. 302:348-355.
  2. Zhu, H., G. Bannenberg, P. Moldeus and H. Shertzer. 1994. Arch. Toxicol. 68:582-587.
  3. Ohashi, T., A. Mizutani, A. Murakami, S. Kojo, T. Ishii and S. Taketani. 2002. FEBS Letters 511:21-27.
  4. Brown, M., F. Miller, W.-G. Li, An. Ellingson, J. Mozena, P. Chatterjee, J. Engelhardt, R. Zwacka, L. Oberley, X. Fang, A. Spector and N. Weintraub. 1999. Circ. Res. 85:524-533. (Measurement of fluorescence)

Materials: HBSS **MUST** have calcium and magnesium or cells will retract.

**WORK IN DARK—CM-H<sub>2</sub>DCFDA is extremely light sensitive!**

CM-H<sub>2</sub>DCFDA, MW: 577.8 g/M

Molecular Probes C-6827, Lot 3401-3

50 µg in vial

Dissolve in 8.6 µl DMSO to give 5.8 µg/µl or 10 mM stock

577.8 g/M X 0.01 M/l = 5.8 g/l or 5.8 µg/µl = 50 µg/X = 8.6 µl

Procedure:

1. Rinse cells 2X with HBSS **WITH** calcium and magnesium.
2. Add 8 µl CM-H<sub>2</sub>DCFDA 10 mM stock + 8 mls HBSS , then add 1 ml to each well of 6-multiwell dish (or to each 35 mm-diameter dish).
3. Final concentration of CM-H<sub>2</sub>DCFDA will be 10 µM.

**NOTE: CM-H<sub>2</sub>DCFDA is EXTREMELY light sensitive--keep in DARK**

4. Leave CM-H<sub>2</sub>DCFDA 10-30 min **IN DARK**.
5. Add Ang II for time course 0-15 min (5 µl Ang II stock into 1 ml HBSS in dish/well). Either rinse out CM-H<sub>2</sub>DCFDA or add Ang II stock directly to dish/well.
6. Wash cold HBSS.
7. Observe DCF staining in confocal microscope. **DO NOT USE Hg LAMP** to focus cells. DCF fluorescence **INCREASES** with the light. Use 488 nm excitation line and 515/30 bandpass emission filter.

**NOTE: Laser light itself produces DCF-DA. Thus DO NOT KEEP LASER ON SPECIMENS FOR EXTENDED PERIODS.**

CM-H<sub>2</sub>DCFDA is widely used to measure oxidative stress in cells. CM-H<sub>2</sub>DCFDA is resistant to oxidation, but when taken up by cells, is de-acetylated by intracellular esterases to form the more hydrophilic nonfluorescent reduced dye dichlorofluorescein DCFH, which then is rapidly oxidized to form a two-electron oxidation product, the highly fluorescent DCF in a reaction with the oxidizing species (H<sub>2</sub>O<sub>2</sub>).