### References

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

**WORK IN DARK—CM-H$_2$DCFDA is extremely light sensitive!**

CM-H$_2$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$_2$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$_2$DCFDA will be 10 μM.

**NOTE:** CM-H$_2$DCFDA is EXTREMELY light sensitive--keep in DARK

4. Leave CM-H$_2$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$_2$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$_2$DCFDA is widely used to measure oxidative stress in cells. CM-H$_2$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 dichlorofluorescin 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$_2$O$_2$).