PROTOCOL for **LIVE/DEAD** STAINING :: dcjoyner@lbl.gov

Supplies:

-LIVE/DEAD BacLight Bacterial Viability Kit (kit contains dyes and mounting oil)
Molecular Probes cat # L-7007 go to : www.probes.com for refs, info etc.
-Black Polycarbonate Membranes 25mm 0.2um (available through VWR)
Whatman Nucleopore cat # 110656
-Epifluorescence microscope with a **GFP/FITC** (ex:480nm em:500nm) and **rhodamine** filter (ex:490nm em:635nm) (green =live cells red=dead cells)
-25mm Filter Holder w fritted glass support (VWR # 26316-690), 15ml glass funnel (VWR # 26316-694), Aluminum clamp (VWR # 26316-700), Silicone stopper #5 (VWR # 26316-702) and a Filtering Flask (VWR # 26316-736).

Stock Solutions:

-**Stain**: prepare a 200X dilution of each dye (Component A and Component B) in filter sterilized water
(ie> 10ul A + 10ul B + 1980ul f.s. H2O)
store solution for up to 2 weeks at 4C in the dark
-**Buffer:** we use 50mM filer sterilized $KHPO_4^{-2}$ buffer
(but any bacterial diluent will do… the object is to not lyse the cells or damage the membranes)
for soils you have the option of using 0.1% NaPPi

Method:

-prepare **serial dilutions** of sample in buffer
(for soils usually 10-4 or 10-3) each sample is different,,, and with a new sample it is best to prepare 3 dilutions, your target dilution and one above and one below

*in selecting your optimal dilution, you want a dilution that will provide you with approximately 20-30 cells/field of view. In addition, you want to dilute the sample enough so that there is no overlap of soil particles in a field of view, which might cover cells.
*for soils with microbes that adhere strongly to the soil particles, prepare the first serial dilution in the NaPPi buffer (this will weaken the polar bonds, releasing many of the microbes from the soil particles) and shake for 15m at 100rpm.
*be sure that sample is well mixed and that serial dilutions are accurate.

-transfer a 500ul aliquot of the final dilution to a sterile tube
-add 120ul of stain, incubate in the dark for 15 min.
-after incubation, wash off all unbound dye by adding 5-10ml of your buffer to the tube
-vortex gently and filter onto Black Polycarbonate membrane (using filtration apparatus)
-mount membrane onto coverslip and view with epifluorescence microscope at 100X
** I like to place a drop of oil onto the slide and put the coverslip over the drop (perpindicular to the slide) to spread the oil out flat… when you are ready to place your membrane on the slide,,, remove the coiverslip by sliding it off to the side…. This way you have a nice thin film of oil that will serve to hold the membrane flat on the slide. Place a drop of oil on the membrane and use the same coversalip to cover the membrane

view your samples quickly as photobleaching occurs. Keep them out of the light if there is a delay between preparation and counting. GOOD LUCK !!