**Staining of yeast cells with DHR**

Last updated on 2012 June 10, Hong Qin

**Background**

The stock solution of Dihydrorhodamine 123 (DHR) is 25mM in DMSO, made by add 1.155ml DMSO to 10mg DHR (Invitrogen D632), which is about 8.5mg/ml.

DHR can be measured in FL1 (530nm+/15nm) in FACSCaliber2. DHR is green.

Rhodamine 123, Ex @ 507 nm, Em @ 528 nm.

DHR detects intracellular H2O2 in yeast.

**Procedure (largely based on Mesquata PNAS paper, and 2012April9 DHE procedure)**

1. Grow yeast cells in a choice of media overnight.
2. Restage cells to log-phase and grow for 2 hours to make sure that most cells are in log-phase.
   1. For log-phase, it is safe to restage the culture in desired media on the same day. Gupta usually does 1:10 dilution for YP2%D cutures (0.5 ml culture to 4.5 ml of fresh media) and 1:6 for YP0.5%D cultures.
   2. Tip: It is critical to keep tracks of different cultures from different dates, media, and strains. A good practice is to use different colored labels and pens for different dates, media, and strains.
3. Spin down 2x1ml of cells, wash with 1ml fresh media, and resuspend in 1ml fresh media.
   1. Make sure the same media are used, especially for Calorie Restriction experiments.
4. Point-sonicate the cells @ level 2, quick-push button 2 times.
5. Make master mix of DHR staining resolution using the PBS
   1. For one reaction: PBS =0.19 ml, 25mM DHR= 0.5ul (for 10ul cells)
   2. Times the above mix by (the number of reactions + 1)
   3. The final DHR concentration is 25000\*0.5/200=62.5uM.
   4. Note: DHR should be kept on ice and shielded from light.
6. Split the master mix to the number eppendorf tubes, label with strain, incubation time, and other necessary information.
   1. Make sure that “no stain control”, and “single-stained”, are also prepared.
   2. Add DMSO to no-stain and single stain control. (This is often skipped unless DMSO really affect ROS signals)
7. Add 10ul of cells to corresponding tube with master mixes.
8. 30C incubation in dark for 1.5 hours (covered with aluminium foil)
   1. For time series, back-calculate the incubation length, so all the sample will finish incubation at the same time.
   2. Note: Mesquita use 26C for 1.5 hours, but Rowe use 30C for 2 hours.
9. Spindown, resuspend in 1ml PBS, keep them in boxes wrapped with aluminum foils.
10. Proceed to FACS analysis

**BD FACS Calibur usage**

**Caliburation of BD FACS Calibur using CaliBRITE Beads**

1. Check sheath tank and waste tank. Add sheath and empty waste is necessary.
2. Switch fluidics to pressuried.
3. Turn on BD FACS Calibur machine
4. Turn on computer and login.
5. While the Calibur is warming up (ususaly for at least 15 minutes?), prepare the beads.
6. Prepare two 12x75mm Falcon polystyrence tubes, label them as TubeA and TbueB
7. Add 1ml of sheath fluid to tubeA
8. Add 3 ml of sheath fluid to tube B (If PerCP-Cy5.5 is used, BD recommends bead dilution buffer should be used. However, we have ignored this)
9. Gently mix the CaliBRITE beads
10. For 3 color calibration, add 1 drop of Unlabaled beads to A; 1 drop of Unlabled, FITC, PE and PerCP each.
11. Keep these prepared beads suspensions ice and shield from light.
12. Run FACS Comp
13. … …

**Caliur measurement in Cell Quest (see Calibur protocol).**

1. Plot template
2. Instrument settings

**Notes:**

\* Paul Doetsch lab label yeast cells with DHE in YPD because they found cells metabolize well in YPD. (If cell mainly metabolized on endogenous carbonhydrates, this should not be a problem).

\* Frank Madeo label DHE with 10 minutes of incubation at 30C.

\* In BD FACSCalibur, I found DHE can be detected in FL2 (585/42nm) and FL3 (680LP), but not in FL1 (530/30nm) (see note on 2012 Feb 22).

**References:**

**In Madeo 1999:**

Free intracellular radicals were detected with dihydrorhodamine 123, dichlorodihydrofluoresceindiacetate (dichlorofluorescin diacetate), or dihydroethidium

(hydroethidine; Sigma Chemical Co.). Dihydrorhodamine

123 was added as **5 ug per ml** of cell culture from a **2.5-mg/ml stock** solution

in ethanol (**HQ: This is 1:500 dilution, and Ethanol can affect lifespan**) and cells were viewed without further processing through a rhodamine optical filter after a **2-h** incubation.

Dichlorodihydrofluorescein diacetate was added ad-10 ug per ml of cell culture from a 2.5 mg/ml

stock solution in ethanol and cells were viewed through a fluorescein optical filter after a 2-h incubation. Dihydroethidium was added ad-5 ug per ml of cell culture from a 5 mg/ml aqueous stock solution and cells were viewed through a rhodamine optical filter after a 10-min incubation. For

flow cytometric analysis, cells were incubated with dihydrorhodamine 123

for 2 h and analyzed using a FACS® Calibur (Becton Dickinson) at low

flow rate with excitation and emission settings of 488 and 525–550 nm (filter FL1), respectively.

**Rowe thesis** use 25ug/ml DHR, 50ug/ml DHE, incubation for 2 hours. Rowe used much higher concentration probably due to the mutagenetic process involved.

In **Mesquita** PNAS paper, **DHR** was added to a final concentration of **15 μg/mL** and cells

were incubated for **90 min at 26 °C.** DHE signals can be captured by FL3 (>670nm) in FACSCaliber2. So, this is red. Intracellular superoxide anions were measured using dihydroethidium (DHE) (Molecular Probes). Aliquots of cells were collected at indicated time points and DHE was added to a final concentration of 5 μM from a 5-mMstock in DMSO. After incubation for 10 min at 30 °C, cells were washed once with 0.5 mL PBS, resuspended in 50 μL PBS, and added to 1 mL PBS. After

briefly sonicating the suspension, DHE signals were measured

using a FACSCaliber2 flow cytometer (BD-Biosciences) with

a 488-nm excitation laser. Signals from 25,000 cells/sample were

captured in FL3 (>670 nm) at a flow rate of 5,000 cells/s. FACS

measurements of DHR signals presented in Fig. 2 were measured

similar to the DHR measurements described for Fig. 1 except that

a FACSCaliber flow cytometer was used to capture signals in FL1

(530 nm ± 15 nm) from 25,000 cells/sample at a flow rate of 5,000

cells/s. Data collected with the FACSCaliber2 flow cytometer were

processed with Flowjo software (Tree Star) and quantified with

WinList software (Verity Software House)

Q FL4 calibration