MURI Staining Protocol

This is not set in stone, as I'm waiting for the appropriate light fixtures to be installed on the scope.

Background

We want to assess microbial viability. Traditionally this is thought to be about differentiating between dead and potentially viable cells. However, given the nature of this project and the focus that it puts on starvation, it is important to assess microbial activity as well as viability.

This will be done through the use of three dyes.

1) eFluor 660

- Permeates dead cells, giving you a background fluoresence.

2) DAPI (4',6-diamidino-2-phenylindole)

- Binds to AT-rich regions of DNA.

3) CTC (5-cyano-2,3-ditolyltetrazolium chloride)

- CTC is a tetrazolium salt that is reduced to formazan dye by the electron transport system.

While we know that the dyes work in combination, we have yet to test this specific protocol on the cultures, as we are waiting for the appropriate scope parts to arrive. Protocol probably will need tweaking for each strain and each treatment, etc.

Dye	Excitation (nm)	Emission (nm)	Laser (nm)	notes
eFluor 660	633	660		
DAPI	358	461	UV	
CTC				

1) Light Microscopy

Live/dead staining

- Aliquot 1 mL samples of your culture to seperete Eppendorf tubes.
- Stain samples with 1 uL eFluor 660 per mL. Incubate for 30 minutes at 4C.
- There could potentially be overstainning (this needs to be tested). If that occurs in your samples, wash some of the stain out before proceeding, as follows:
    - Centrifuge samples at 10,000 RPM for 10 min.
    - Pipet ~500 mL of supernatant (adjust according to need)
    - Resuspend pellet.

Respiration Staining

- Make 10 mM aliquots of CTC dye.
- Add 500 uL to sample, giving you a final CTC concentration of 5 mM.
- Incubate for 30 - 60 min. at 37C.

Fixation

- Add 13.5 uL filtered 37% formaldehyde to each sample. Vortex for 5 sec.

DNA Staining

- Add DAPI stain so that you have a 4.5 mM final concentration in your sample (depends on your aliqout solution)
- Incubate for 10 min. at 37C.

Filtering

- Wash filtration column with 70% EtOH or 10% bleach, then rinse with ePure H_2O. Rinse between each sample.
- Place a 0.8 micron backing filter onto the glass frit.
- Wet the filter and pull down excess H_2O
- Place a 0.22 micron black polycarbonate filter on top of the backing filter.
- Add ~ 5 mL ePure H_2O to your filtration column and transfer an aliquot of your sample to the filtration column (assess volume by initial density of culture).
    - Record this volume so you can back-calculate cells/mL
- Pull sample down onto filter with low pressure (< 10 Hg)
- Transfer the filter onto a standard glass slide using forceps.
- Place a drop of 4:1 Citifluor-Vectisheild antifade solution onto a cover slip

- Once dry, place a coverslip over the slide and remove excess oil.
- Store slides for transfer to scope

2) FLow Cytometry

A part of my research is looking at cell activity and sorting cells based on a certain level activity. Therefore, I have adapted the above protocol into one appropriate for flow cytometry analysis.

CTC has not yet been calibrated for the IU Flow Cytometry Core facility and I am working on generating samples for them.

Protocol is same as above, with minor alterations. Working with FC facility.