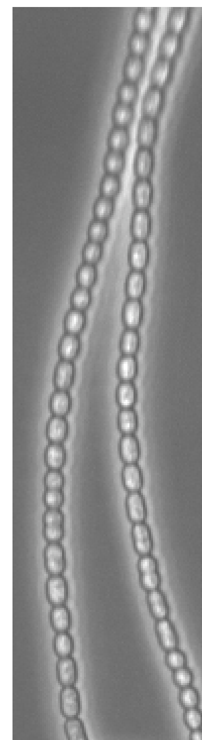
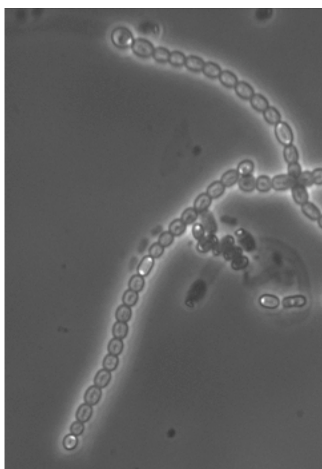
Inducing akinetes with or without heterocysts (7/07)



Purpose: Epifluorescence microscopy of promoter-reporter strains. Differentiating akinetes from other cell types is difficult due to the reversibility of akinete differentiation; therefore, localization about halfway between heterocysts supplies associative evidence when combined with cell morphology.

Start with vegetative cells composed of filaments hundreds of cells long. To obtain such a culture, cells must be grown through 2 bottle changes under 8-12 PFD light @ 25 deg. C. Inoculate cultures to about 1 ug/mL chla. Any lower concentration will break filaments due to high light stress response. An image of healthy vegetative cells is shown at the right. All cells should have the same morphology. If they do not, the culture is not ready for differentiation. I suggest maintaining your cultures between 1-10 ug/mL chla in appropriate antibiotics at all times. When you are ready to induce differentiation, grow a culture without antibiotics to 6-8 ug/mL chla (This should be late log…please look at a *N. punctiforme* growth curve from cultures in the same incubator in which you are growing your cultures.)

Starving the cells for easily used combined nitrogen compounds forces heterocyst induction to provide vegetative cells with nitrogen. To remove combined nitrogen (these are the ammonia (A) and nitrate (N) in MAN), wash the 50 mL vegetative (observe in microscope to ensure >100 cell filaments) culture with 50 mL of either water, or better, A&A/4 +Pi +MOPS 3 times spinning @ < 3 kxg >2 min. Vortex or shake culture in each fresh wash. Always use wide bore or glass pipettes to transfer cells and avoid strand disruption. Deformed cells have been observed after washing with water, so try both water and A&A to determine the method that works best for you. After washing, re-suspend pellet in A&A/4 +Pi +MOPS and return it to the same incubator in which it was cultured. Allow 24-48 hours for heterocyst differentiation.



After 24-48 h induction the culture should contain aggregated cells in clumps. These clumps contain heterocysts. Transfer to 50 mL falcon tube and allow the culture to settle for 15 min.. The top layer should contain hormogonia. The dense bottom layer should contain filaments with heterocysts every 10-30 cells. Check for hormogonia and heterocysts (under phase contrast, heterocysts contain bright cyanophycin granules at left) under the scope. Decant (remove) the upper hormogonia layer with a pipette.

Now that you have ensured heterocyst formation and have removed contaminating hormogonia, phosphate starvation will force akinete differentiation starting about midway between the heterocysts. Wash as before, but this time use A&A/4 **-Pi** +MOPS or water, and resuspend in 50 mL of A&A/4 **-Pi** +MOPS with 80 μL of 0.01x filter sterilized +Pi stock added. Check for cell damage under scope. Place culture @ room temp 22 deg. C. 3-8 PFD with low shaking rate. Observe every 2 days and take pictures using the 40x or 100x objectives. Akinetes are granular, more rounded, and start forming about midway between heterocysts as in the picture below. Note that heterocysts do not fluoresce under Texas red and late akinetes are dimmer. A plasmid only control (no insert to drive the reporter) is required for comparison in any promoter reporter experiment.

1. Wash solution for induction of heterocysts:
    a. Make liter quantities of AA/4 media as described in the green book in 1 liter flasks and autoclave.
    b. Autoclave (cycle 4), cool to room temperature, add MOPS buffer (pH 7.8) from filter sterilized concentrated stock to a final concentration of 5 mM. Mix and used this for washing cells. Add only MOPS to AA/4 liquid medium for induction of heterocysts.
2. Wash solution for induction of akinetes:
    a. Make liter quantities of AA/4 in 1 liter flasks, but **do not add the +Pi stock component** of the media. Use acid washed glass only (removes phosphate).
    b. Autoclave (cycle 4), cool to room temperature, add MOPS buffer (pH 7.8) from filter sterilized concentrated stock to a final concentration of 5 mM. Use this buffered –N, -Pi media for washing cells. This is the same medium into which you will place the washed cells for induction of akinetes in the presence of heterocysts (so be sure make some 50 ml media in 125 ml flasks prior to experiment…remember to used acid washed flasks only for –Pi media).
3. 0.01x –Pi added as 80 μL/ 50 mL culture:
    a. Dilute 0.5 mL of +Pi stock component to 50 mL in distilled water.
    b. Filter sterilize to 0.2 μm.
        a. Add 80 μL of this to each 50 mL culture after washing away phosphates.

Differentiating akinetes only

The purpose here is to make akinetes for purification. This may be desired to characterize the phenotype of a mutant as compared to wild-type akinetes, or to assay proteins from only akinetes. In this case, heterocysts are undesirable, and will be eliminated by:

1. Grow up cells in the presence of MAN to late exponential/early stationary as above with visual confirmation. The presence of combined nitrogen will inhibit heterocyst induction. DO NOT induce heterocysts by washing with –N media.

2. Wash cells including nitrate in the –Pi wash and final incubation for akinetes. In this case make liter amounts of AA/4 with MN (MOPS and nitrate; 5mM each final concentration) and re-suspend washed pellet in flasks containing the same media.
3. Incubate as before in low light with slow shaking.

Note: ALWAYS include a wild-type akinete induction as a control for comparison to any mutant. Akinete induction will occur randomly throughout the filament in about 2 weeks.

Note that heterocysts do not fluoresce under Texas red and late akinetes are less fluorescent than vegetative cells. This is due to total or partial degradation of light harvesting physobiliproteins which fluoresce under this filter set.