FVIC Protocol

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October, 2011

Purpose: This protocol describes how to pellet bacterial cells onto grids and stain them so that viruses can be visualized within the cells using transmission electron microscopy. Data obtained from these grids can be used to calculate the frequency of visibly infected cells (FVIC), the frequency of infected cells (FIC), the fraction of mortality due to viral lysis (FMVL), and burst size.

Materials required:

- glow discharge apparatus
- ultracentrifuge and rotor capable of 55000 x*g*
- pollyallomer ultracentrifuge tubes with epoxy in the bottom to provide a flat surface
- EM grid-grade forceps
- EM grids (formvar coated 200 mesh copper grids)
- paper dividers from between commercially packaged filters
- double-sided tape
- razor blade
- uranyl acetate
- ultra-purified water such as Milli-Q water
- petri dish
- 4x 2 ml microcentrifuge tubes with screw caps
- 4 ml plastic culture tube with cap
- small stir bar
- stir plate
- filter paper (cut into wedges)
- 0.02 µm syringe filter and syringe (3-5 ml)
- lab coat, respiratory protection (mask), eye protection
- timer
- transmission electron microscope

Preparing the grids:

1) On the same day of grid prep, use glow discharge to render grids hydrophilic

2) Cut a circle out of a paper filter divider such that it has a diameter slightly smaller than the interior diameter of the ultracentrifuge tubes

3) Cut small rectangles (~3 mm x 1 mm) of double-sided tape using a razor blade

4) Attach the discharged grids (shiny-side-up) to the circle of paper using the double-sided tape such that *only the edge* of the grids contacts the tape (Figure 1)

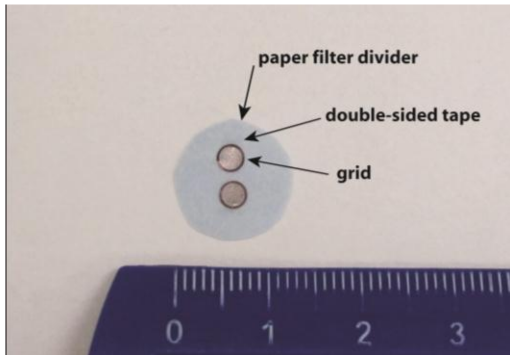

Figure 1. Grids attached to the paper filter divider with double-sided tape.

5) Insert the paper with the attached grids into the ultracentrifuge tube grids-side-up (Figure 2)

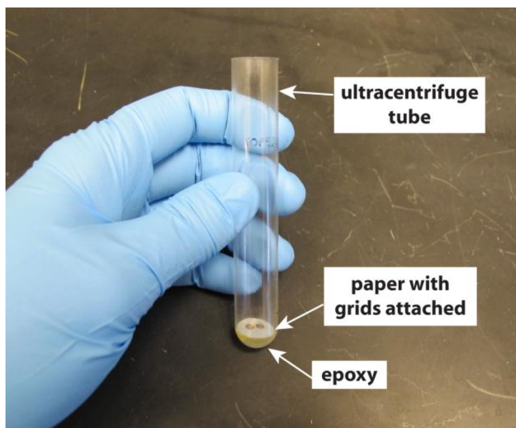

Figure 2. Grids inserted into the bottom of the ultracentrifuge tube.

6) Add your sample to the ultracentrifuge tube ensuring that there are no air bubbles under the circle of paper
   *Note: ca. 20 ml of sample is required for samples with bacterial concentrations of 10^7 ml^{-1}. Adjust the sample volume according to the bacterial concentration in your samples.

7) Centrifuge samples using the following conditions (you can use a different rotor as long as it can reach the appropriate g-force):
   Rotor: Beckman SW 40 Ti
   Temperature: 4ºC
   Time: 1 hr
   Speed: 20000 rpm (average force = 55000 x*g*)
   *balance the rotor *exactly* with another sample or dummy sample

8) After centrifugation, you can remove the sample and add more sample for additional centrifugation runs to achieve the required volume

9) Make 2 ml of 0.5% uranyl acetate with Q-water in a 4 ml culture tube using a stir bar to mix the sample on a stir plate for ~30 minutes to 1 hour (do not turn on the heat!)
   (0.01 g uranyl acetate + 2 ml Q-water = 0.5% uranyl acetate)
   *Caution: uranyl acetate is toxic so read the MSDS and use a lab coat, eye protection, respiratory mask, and gloves when handling it in powdered form

10) Filter the uranyl acetate solution through a 0.02 µm syringe filter into a 2 ml screw-cap tube
   - this removes any particles of uranyl acetate that have not fully dissolved

11) Fill 3x 2 ml screw-cap tubes with Q-water

12) Using tweezers, remove the piece of paper with the attached grids from the ultracentrifuge tube and place in a petri dish

13) Using tweezers, remove a grid from the piece of paper (make sure the tape is not still attached to the grid)

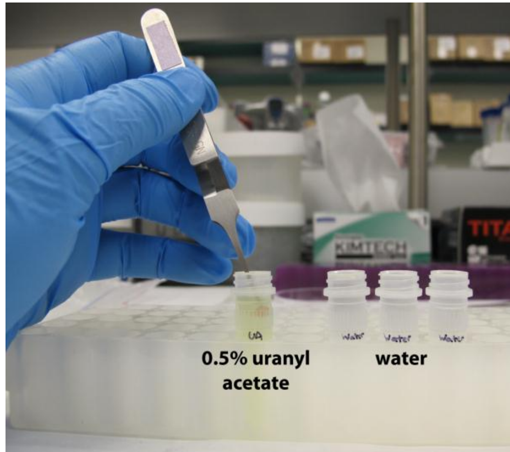

Figure 3. Immersing the grid in the uranyl acetate solution.

14) Immerse the grid into the 0.5% uranyl acetate solution for 30 seconds (Figure 3)

15) Immerse the grid into the 1st tube of Q-water for 10 seconds

16) Immerse the grid into the 2nd tube of Q-water for 10 seconds

17) Immerse the grid into the 3rd tube of Q-water for 10 seconds

18) Wick away liquid from the grid using a wedge of filter paper placed at the edge of the grid (Figure 4)

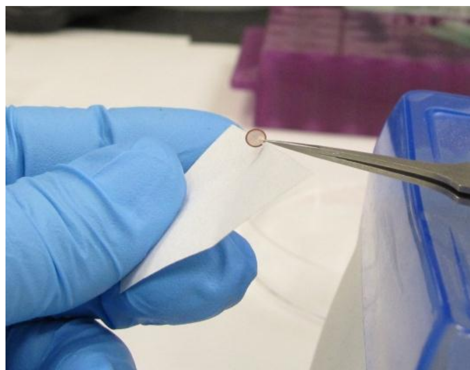

Figure 4. Wicking liquid from the grid.

19) Place the grid in a grid box and allow to dry overnight, then store the grid in a desiccator until analysis (do not put wet grids in a desiccator immediately as this can create "cracking" of the stain)

Grid analysis:

1) Using a transmission electron microscope with 100 kV accelerating voltage, examine a grid at ~40000X

2) Look at 800 cells per grid
   - count how many cells have >5 viruses assembled within the cell (Figure 4)
     - the "greater than 5" rule prevents you from mistaking cellular structures or extracellular viruses from intracellular viruses
   - take a digital micrograph of all visibly infected cells

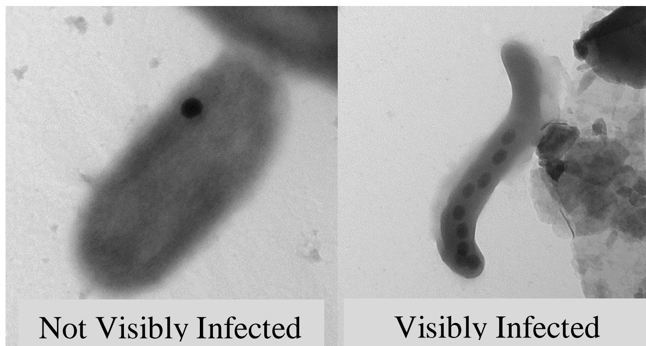

Figure 4. Examples of cells that would be counted as visibly infected and not visibly infected.

3) Calculate the frequency of visibly infected cells (FVIC):

$$FVIC = \frac{(number\ of\ visibly\ infected\ cells)}{(total\ number\ of\ cells\ examined)}$$

4) Calculate the frequency of infected cells (FIC)

$$FIC = (7.1 \times FVIC) - (22.5 \times FVIC^2)$$

5) Calculate the fraction of mortality from viral lysis (FMVL)

$$FMVL = \frac{[FIC + (0.6 \times FIC^2)]}{[1 - (1.2 \times FIC)]}$$

6) Determine burst size
   - Using the micrographs of visibly infected cells, count how many viruses are present inside of *cells that are full of viruses*
   *Caveats: - some cells can burst before they are full of viruses (thus, this is an overestimate of burst size)
   - it may be impossible to count the total number of viruses within larger cells because they are overlapping one another (thus, this is an underestimate of burst size)

7) Other information that can be obtained using ImageJ or other image analysis software:
   - dimensions of infected cells
   - dimensions of intracellular viruses

Applicable References:

Proctor and Fuhrman (1990) – The original description of the FVIC method.

Binder (1999) – The equations used to calculate FIC and FMVL, and the assumptions inherent in those equations.

Brum et al. (2005) – An example of a use of the FVIC method to investigate viral induced mortality of bacteria in an aquatic ecosystem.

Binder, B. (1999) Reconsidering the relationship between virally induced bacterial mortality and the frequency of infected cells. *Aquatic Microbial Ecology* **18**: 207-215.
Brum, J.R., Steward, G.F., Jiang, S.C., and Jellison, R. (2005) Spatial and temporal variability of prokaryotes, viruses, and viral infections of prokaryotes in an alkaline, hypersaline lake. *Aquatic Microbial Ecology* **41**: 247-260.
Proctor, L.M., and Fuhrman, J.A. (1990) Viral mortality of marine bacteria and cyanobacteria. *Nature* **343**: 60-62.