

Preparation and application of fluorescently labeled virus particles

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Abstract

Tracing the fate of individual host cells and viruses is a challenging problem for microbial ecology. The emergence of a new assay, using fluorescently labeled viruses (FLVs), offers the promise of a quick and easy method for monitoring host dynamics and virus decay. Using FLVs as probes (FLVPs) for host cells, an assay was optimized for use with SYBR Green I–stained phages and was shown to be an efficient and reliable method for detection of a strain of *Vibrio* sp. Various microcosm experiments were conducted that demonstrated the utility of the FLVP assay in resolving ecological interactions at the community level. The assay was also used to show that FLVPs, at high enough multiplicities of infection, can directly inhibit viral infection by “titering out” or “coating” the host’s cell surface receptors. FLVs can also be used as tracers for studies of virus production and decay. The approach is mathematically similar to the isotope dilution technique, employed in the past to simultaneously measure the release and uptake of ammonium and amino acids. The method can be used to determine rates of viral degradation, production, and turnover for investigations of microbial food webs in aquatic systems.

Introduction

Almost two decades ago, pelagic marine viruses were first reported to exist in high numbers in the marine environment, exceeding the typical abundance of bacteria (Bergh et al. 1989, Proctor and Fuhrman 1990). Since then, they have

been demonstrated to be agents of significant mortality of heterotrophic bacteria, cyanobacteria, and phytoplankton (Fuhrman 1999, Wilhelm and Suttle 1999, Suttle 2007). Specifically, it has been shown by a variety of researchers that viruses are capable of causing up to 50% of the bacterial mortality in a range of aquatic environments (e.g., Fuhrman and Noble 1995, Steward et al. 1996, Guixa-Boixareu et al. 1996, Weinbauer and Höfle 1998). The variability of the impact that viruses have on bacterial assemblages can be high, even over short periods in the same study area (Bratbak et al. 1996, Steward et al. 1996, Bongiorni et al. 2005). With their influence on aquatic microbial populations, viruses appear to have the potential to affect the flow of energy and matter in marine ecosystems. For example, viral infection of bacterial cells, and subsequent cell lysis, has been suggested to result in a “short circuit” in the microbial loop, where recycling fuels bacterial production and respiration and reduces the amount of organic matter available to macroorganisms (Fuhrman 1992, Thingstad et al. 1993). Accurate measurements of viral production and turnover are important to accurately assess their impacts on microbial food webs, carbon cycling, and trophic dynamics.

Epifluorescence microscopy, combined with the use of fluorescent stains such as DAPI, SYBR Green I, and Yo-Pro I, is a well-documented approach for the enumeration of bacteria and viruses (Hennes and Suttle 1995, Weinbauer and Suttle 1997,

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Noble and Fuhrman 1998, Weinbauer et al. 1998, Patel et al. 2007). Tracing the fate of individual host cells and viruses, however, has historically been a challenging problem for microbial ecology. Fluorescently labeled viruses (FLVs) can be used as both probes of host cells of interest and tracers of viral dynamics. In this chapter, we detail both uses of FLVs, using examples of how the methods can be applied where appropriate.

Using FLVs as probes (FLVPs)—The use of fluorescent labeling in aquatic ecology developed rapidly around the early 1990s, especially in studies on the dynamics of grazing and bacterivory using fluorescently labeled bacteria, algae, viruses, and even synthetic microspheres (Ruble and Galle 1989, Sherr et al. 1991, González and Suttle 1993, Epstein and Rossel 1995). FLVs were originally created using the first-generation nucleic acid-binding fluorochromes, such as acridine orange and DAPI, which had been previously used to visualize individual viruses (Daley and Hobbie 1975, Coleman et al. 1981, Hara et al. 1991). Shortly thereafter, Hennes et al. (1995) described a new use for FLVs, using second-generation cyanine-based dyes, as specific probes (FLVPs) for single species of prokaryotes. The fluorescent labeling did not hinder the protein-protein interactions required for viral attachment to the host, and the species-specific nature of viruses allowed for the in situ detection of the host and the characterization of abundances/dynamics in mixed microbial assemblages. They also showed that multiple FLVPs of varying colors could be used to detect multiple species in the same samples. Since the introduction of this method, FLVPs have been used to trace different bacteria in natural settings, such as phage-sensitive bacteria in biofilms (Doolittle et al. 1996) and the polyphosphate-accumulating bacteria *Microcystis* in sludge samples (Lee et al. 2006). Significant interest in FLVPs has also been shown for tracking human pathogens such as *Salmonella* (Mosier-Boss et al. 2003) and *Escherichia coli* (Goodridge et al. 1999, Tanji et al. 2004, Kenzaka et al. 2006). The focus of the present chapter is to describe the FLVP method, using a slightly simplified protocol with a newer fluorochrome, and to expand on the work of Hennes et al. (1995) by further illustrating the use of FLVPs in microcosm studies.

Using FLVs as tracers—Rates of virus production and removal can be determined using calculations previously used for the isotope dilution technique to measure rates of release and uptake of amino acids or dissolved ammonium by using radioisotopes or stable isotopes, respectively, as tracers (Blackburn 1979, Glibert et al. 1982, Fuhrman 1987). The initial use of this approach is discussed at length in Noble and Fuhrman (2000). The FLVs are similar to labeled molecules (e.g., radioisotopes) when used as tracers and when added to water samples at low levels (<10% of the ambient viral concentration). Processes of virus decay and clearance decrease the number of FLVs and unstained viruses in relative proportion to the total virus abundance. New virus production produces only unlabeled viruses, however, thereby diluting the initial pool of FLVs.

Using the rate of change of both labeled and unlabeled viruses over time, rates of virus production and removal can be calculated. The method is particularly useful for measuring virus production and removal in oligotrophic areas, where radiolabeling approaches such as that outlined by Steward et al. (1992a, 1992b) are difficult. The method permits simultaneous determination of rates of virus production and removal using epifluorescence microscopy. The data generated from the approach can be used to populate conceptual and numerical models of virus production and decay. This approach can be effectively used in oligotrophic and mesotrophic aquatic systems, but is not intended for use in highly productive aquatic systems (such as eutrophic estuaries) where high concentrations of total suspended solids and detritus are found (see “Assessment”).

Materials and procedures

FLVP assays—Viral and bacterial isolates, propagation and preparation of stocks: The virus-host system used for the FLVP experiments was *Vibrio alginolyticus* strain PWH3a, a marine heterotrophic bacterium, and its phage PWH3a-P1, a species-specific dsDNA virus of the *Myoviridae* family. Both strains were originally isolated from the coastal waters of the Gulf of Mexico (Suttle and Chen 1992). The host was maintained as a -80°C glycerol stock to minimize any long-term culturing effects and was cultivated at 30°C with agitation (120–200 rpm) using Marine Luria-Bertani broth (MLB) (0.5 g L⁻¹ each of casamino acids, peptone, and yeast extract, 0.3% vol/vol glycerol, in 25 psu ultrafiltrate base). The virus was amplified using the plate lysate/liquid elution method (Suttle 1993), substituting ultrafiltered (virus-free) seawater for sterile media as the eluting agent. The eluant from multiple plates was pooled into a 50-mL centrifuge tube and spun at $\sim 4000g$ for 20 min to remove large debris. The supernatant was then collected and filtered through a 47-mm-diameter, 0.22- μm -pore-size Durapore (Millipore) membrane to remove any remaining host cells. The final viral stock was titered by plaque assay (Suttle 1993) and kept at 4°C in the dark until needed.

The following materials and equipment are required for the preparation of FLVPs:

- amplified, concentrated virus stock (preferably $\geq 10^{10}$ viruses mL⁻¹); calculate amount of stock needed using the following guide: 1 tube (1.7 mL) of stock = 50 μL of FLVPs \rightarrow 1 μL FLVPs/slide = 50 slides;
- Screw cap 1.7-mL microcentrifuge tubes (often listed as 1.5 mL);
- 0.02- μm filtered water or appropriate filtered seawater medium for resuspensions; use the latter if the virus is destroyed by freshwater;
- SYBR Green I dye working stock (Molecular Probes);
- RC80 Beckmann (or similar) ultracentrifuge with an SW40 (or similar) swinging-bucket rotor; alternatively, use a rotor capable of directly accepting microcentrifuge tubes in a centrifuge capable of reaching $>50,000g$;

- epifluorescence microscope equipped with a 100× oil-immersion objective and a blue-light excitation filter (such as the Olympus U-MWB2/U-MWIB2 filters).

The following procedure is an adaptation of the original FLVP protocol from Hennes et al. (1995) for use with SYBR Green I stain. Normally, this protocol will take 2 days given that the softening of the viral pellet (*see below*) is an overnight step. However, if the viral pellet redissolves quickly, it can be accomplished in 1 day.

1. Pipette 1.7 mL amplified 0.22- μm filtered virus stock into 1.7-mL screw cap tubes. Float the microcentrifuge tubes in long ultracentrifuge tubes (14 \times 95 mm) using water until they are just flush with the top of the ultracentrifuge tubes; balance them to within 1 g. Most standard brands of O-ring screw cap microcentrifuge tubes (10-mm-diameter bodies, 12-mm-diameter screw caps) fit snugly into the larger ultracentrifuge tubes. Alternatively, use a rotor capable of directly accepting microcentrifuge tubes in a centrifuge capable of reaching >50,000g to pellet the viruses.
2. Load the ultracentrifuge tubes into the SW40 rotor and spin them at 133,000g for 1 h. Alternatively, spin at lower speeds in a microcentrifuge-accepting rotor for an equivalent duration (i.e., 2.5 h at 50,000g). A small, whitish pellet should be visible in the microcentrifuge tubes after centrifugation.
3. Recover the microcentrifuge tubes. Remove the supernatant and resuspend the pellet as follows: pipette off 1.5 mL using a P1000; switch to a P100 and gently remove nearly all of the supernatant (remainder usually totals \sim 10 μL); add 40 μL water or seawater medium to bring the volume to 50 μL (remember freshwater versus seawater choice for viral isolate); gently vortex the tubes to disrupt the pellets and place them at 4°C overnight to soften.

Perform all subsequent steps under subdued light since the stain will fade if exposed.

4. Thaw the SYBR Green I, then add 1 μL stain to each concentrated virus tube and incubate for 15 min in the dark.
5. Verify the staining (and monodispersal) of the viruses by pipetting 1 μL of the suspension onto a microscope slide. Add an 18 \times 18 mm coverslip and observe the slide under the epifluorescence microscope. A veritable “sea” of FLVPs should be visible, which will fade nearly instantaneously as you scan from field to field due to the lack of antifade. If you wish to observe the FLVPs for longer periods, add 1 μL of antifade (0.1% *p*-phenylenediamine) to the FLVPs before adding the coverslip.
6. Add 1.65 mL water or seawater medium to each tube and respin as above. The resulting pellet should be slightly orange in the case of SYBR Green I.
7. Remove the supernatant and resuspend as above (completes first wash out of stain).
8. Repeat steps 6 and 7 (completes second wash out of stain).
9. Repeat steps 6 and 7 again (completes third wash out of stain and ends protocol).

Ensure that the resulting FLVPs are monodispersed (as above) after the three centrifugation wash steps. If not, gently vortex the tubes to disrupt the pellets and place them at 4°C overnight to soften. The FLVPs can then be used the following day (day 3) or stored at 4°C for a significant amount of time (*see “Assessment”*).

FLVP assay: The following materials and equipment are required to perform the FLVP assay:

- FLVP stock or 1:100 working stock (diluted in 0.02- μm filtered water or seawater medium);
- 1.5-mL microcentrifuge tubes;
- appropriate host diluent (e.g., sterile seawater medium);
- 0.2- μm Anodisc (Whatman) and 0.45- μm cellulose (backing) filters;
- microscope slides and coverslips (25 \times 25 mm);
- mounting medium containing antifade (50:50 phosphate-buffered saline:glycerol with 0.1% *p*-phenylenediamine);
- epifluorescence microscope capable of 1000 \times magnification and equipped with a blue-light excitation filter (such as Olympus U-MWB2/U-MWIB2 filters).

The following continued adaptation of Hennes et al. (1995) is a result of FLVP assay optimizations for the *Vibrio alginolyticus* PWH3a-phage P1 phage-host system (PHS) for use with SYBR Green I stain. Novices to microscopy would benefit from reading Wen et al. (2004) for details on preparing and storing slides using SYBR Green I stain. This protocol is a modification of the standard slide preparation techniques for bacteria and viruses in aquatic samples (*see Suttle and Fuhrman 2010, this volume*):

1. Prepare a 10-fold dilution series of the culture or natural sample to be enumerated so that \sim 10⁵ cells mL⁻¹ of the target (FLVP-specific species) will be obtained. This will result in a multiplicity of infection (MOI) of at least 3000 viruses per host cell (with an FLVP stock prepared as above from a \geq 10¹⁰ viruses mL⁻¹ initial phage stock). When mixing your host of choice with natural samples (e.g., for microcosm studies), also prepare a background control to check for FLVP attachment to natural cells.

Perform all subsequent steps under subdued light, since the stain will fade if exposed.

2. Add 0.1 mL FLVP working stock to 0.9 mL sample in a microcentrifuge tube for each slide to be prepared and vortex to mix. Conversely, use 1 μL concentrated FLVP stock in 1 mL sample if not using the diluted working stock.
3. Allow up to 30 min for adsorption of FLVPs to target cells. Approximately 15 min was adequate for the PWH3a-P1 PHS, given that the adsorption kinetics of this phage are similar to typical coliphages such as T4; the time depends on the adsorption kinetics of your particular virus-host system and will have to be modified as such.
4. Filter each 1 mL sample onto a 0.2- μm Anodisc filter using a 0.45- μm HA filter for backing.
5. Pipette 10 μL mounting medium onto the surface of a slide, place the filter over the drop, pipette 10 μL mounting medium onto the surface of the filter, and place a 25 \times

25 mm coverslip over the filter. (See “FLV tracer experiments” below for a discussion of mounting media.)

6. Observe the slides under blue-light excitation and count the cells with a fluorescent halo. If a problem with non-specific staining (excess, unwashed stain or leakage from FLVPs) occurs, it will be visible here as diffuse, whole-cell staining instead of the trademark halos.

FLVP microcosm experiments: Seawater samples for the microcosm experiments were obtained from either a seawater holding tank at the University of British Columbia (49°16'N, 123°15'W) or a station in Vancouver Harbor (49°18'N, 123°06'W). All samples, before use in experiments, were screened for the endogenous presence of PWH3a using the FLVP assay.

In the first experiment, where the added (exogenous) bacterium did not dominate the system, PWH3a was added to a final concentration of 6.3×10^6 cells mL⁻¹ in 500 mL of a natural background of prokaryotes at a final concentration of 2.1×10^6 cells mL⁻¹ (~3:1 final ratio PWH3a:natural). The microcosm was enriched with MLB (10% final concentration) and incubated at 20°C with a 14:10 h light:dark cycle. Subsamples were taken at ~24-h intervals for prokaryotic cell counts (see Suttle and Fuhrman 2010, this volume) and FLVP assays. Shortly after the 72-h time point, PWH3a-P1 virus was added to the microcosm at a final concentration of ~10⁵–10⁶ viruses mL⁻¹.

In the second microcosm, where the added (exogenous) bacterium did dominate the system, the PWH3a final concentration was increased to 1.9×10^7 cells mL⁻¹ in 50 mL of a further reduced natural prokaryotic background of 4.4×10^5 cells mL⁻¹ (~40:1 final ratio PWH3a:natural). The microcosm was enriched as before and maintained at the same light level as the previous microcosm, yet the incubation was at the higher temperature of 30°C with aeration. Subsamples were taken as before, and infection of the microcosm was performed shortly after the 48-h time point with the same concentration of PWH3a-P1 virus as above.

FLVP receptor titration experiment: Four flasks of 10 mL sterile MLB were inoculated with PWH3a at a final concentration of 1.4×10^6 cells mL⁻¹. FLVPs were added to three of the flasks at the following MOIs: 0 (uncoated control), 1000, and 10, to attempt to coat the PWH3a bacterium's cell surface receptors and render it resistant to subsequent infection. PWH3a-P1 virus was then added to the three flasks at an MOI of 0.01–0.1. The fourth flask did not contain any FLVPs or PWH3a-P1 and served as the control. Subsamples were taken from the four flasks at 2-h intervals for FLVP assays.

FLV tracer assays—Concentration of viruses and preparation of FLVs for tracer assays: The following steps describe how to prepare each virus concentrate. There are multiple options for many of the steps; in the case where there is more than one option they are noted by capital letters.

Note: All virus concentration steps should be performed either on ice or in a centrifuge held at <10°C, so as to mini-

mize degradation of virus particles during the concentration steps.

1. Collect up to 20 L using either four 5-L Niskin bottles (or other permutation) or by a triple acid- and sample-rinsed bucket into an acid-rinsed 20-L low-density polyethylene carboy.
- 2A. The sample should be filtered at 5 kPa through a 142-mm-diameter, 0.22- μ m-pore-size Durapore filter to remove bacteria and protists. The virus-sized fraction (material between 0.22 μ m and 30 kDa) is concentrated to ~150 mL using a spiral cartridge concentration system (Suttle et al. 1991). Further concentration should be conducted using Centriprep-30 centrifugal concentration units (Millipore) to a final volume of ~5 mL.
- 2B. Alternatively, the sample can be directly concentrated using a tangential flow filtration spiral cartridge concentration system with either a 30- or 100-kDa cutoff (both have shown excellent recovery rates for marine viruses in past experimental procedures; e.g., Suttle et al. [1991], Breitbart et al. [2002]; GE Healthcare, Inc.) and then filtered using a 0.2- μ m Sterivex-type filter (Millipore) to remove unwanted protists and prokaryotes. If desired, further concentration should be conducted using Centriprep-30 or similar centrifugal concentration units (Millipore) to a final volume of ~5 mL.
- 3A. To each of the virus concentrates, SYBR Green I should be added at a final concentration of 2.5% vol/vol and incubated in the dark for at least 8 h at 4°C.
- 3B. To each of the virus concentrates, SYBR Gold (Molecular Probes, Inc.) should be added at a final concentration of 2.5% vol/vol and incubated in the dark for at least 4 h at 4°C.
- 4A. After the staining period, the unbound stain can be rinsed away by adding an equal volume of 0.02- μ m filtered seawater (prepared by filtering fresh seawater from the same location through an acid-rinsed, autoclaved Nalgene filtration unit housing a 47-mm, 0.02- μ m Anodisc filter) to the concentrate and centrifuging it in Centriprep-30 ultraconcentration units at 3,000g for 15 min. This rinse is done three times. Each time, the labeled virus particles are resuspended in a total of 5 mL of 0.02- μ m filtered seawater while reusing the same Centriprep-30 unit.
- 4B. After labeling, the FLVs can be diluted into 1 L of 100-kDa filtrate from the sample site and reconcentrated using tangential flow filtration (TFF). This process is repeated three times to ensure removal of all stain.
5. The final concentrates should be resuspended in a total of 5 mL of 0.02- μ m filtered seawater. To determine the concentration of viruses in the concentrate, 10 μ L concentrate is diluted to a final volume of 2 mL with 0.02- μ m filtered seawater, filtered through a 0.02- μ m Anodisc, and counted by epifluorescence microscopy under blue excitation (Noble and Fuhrman 1998, Patel et al. 2007).

FLV tracer experiments:

1. Collect seawater samples from the desired location. The FLV concentrates should be freshly prepared at each new site and for each new experiment. After determining the concentration of the FLVs in the concentrate, and the ambient concentration of viruses in the seawater, the proper amount of FLV concentrate should be added at tracer levels (<10% of original ambient virus concentration) into sample volumes of no less than 400 mL (the recommended sample volume is 1 L).
2. Designate a control treatment. Several approaches can be used for controls. Formalin-treated (FT) killed controls consist of 0.02- μm filtered formalin added at a final concentration of 2%. Heat-treated (HT) controls are seawater boiled for 10 min and then cooled to ambient seawater temperature. The heat treatment denatures active proteins and enzymes and kills most vegetative bacteria (Karner and Rassoulzadegan 1995). If using TFF, the filtrate can also be used as a control treatment. Any measurable rate of disappearance of FLVs in FT, TFF-filtered, or HT treatments is subtracted from that seen in the untreated bottles. Because SYBR Green I stain fades quickly in sunlight, the samples should be incubated at ambient seawater temperatures in the dark. Experiments can be started at dusk so that the beginning of the experiment is done under simulated in situ conditions.
3. At each time point, total viral abundance and FLV numbers should be determined in duplicate from small volume subsamples (5–30 mL) taken into sterile, 15- or 50-mL polyethylene tubes. The volumes of the subsamples depend on the final concentration of the FLVs, the expected concentration of the native viruses in the sample, and the type of microscope to be used for enumeration. Subsamples are immediately fixed with 1% to 2% (final concentration) 0.02- μm filtered formalin and stored at 4°C. A suggested framework for the experimental approach might be to sample at time 0 h and at 4, 8, 12, and 16 h after initiation of the experiment.
4. Slides should be prepared immediately after sample collection for best results, particularly to avoid fading of the FLV signal. Slides should be prepared according to Noble and Fuhrman (1998) or Patel et al. (2007). Attention should be paid to the mounting solution chosen, as it has been observed that different mounting solutions (*p*-phenylenediamine, ascorbic acid, ProLong) perform differently in different water sample types and different environments (R. T. Noble, data not published). Breitbart et al. (2004) suggested that samples can be held without adverse fading for up to 2 weeks; however, we do not advocate sample storage for longer than a few days unless absolutely necessary.

Note: It is recommended that rates of bacterial production be measured simultaneous to all time points for virus measurements. There are two reasons for this: (1) if measured simultaneously, the researcher can estimate virus production throughout the experiment, rather than relying simply on bac-

terial production estimates from time zero; and (2) bottle effects are common in small-volume experiments such as these. Measurements of bacterial production throughout the experiment will help the researcher to identify times when bacterial production is heightened (or reduced) due to bottle effects.

Calculation of virus production and removal rates: Production and removal rates are calculated from the equations of Glibert et al. (1982) and Fuhrman (1987). The decay constant, k , is calculated as

$$k = \frac{\ln\left(\frac{R_0}{R_t}\right)}{t} \quad (1)$$

where t is the incubation time and R_0 and R_t are the ratios of labeled to unlabeled viruses at time 0 and time t , respectively. The first two time points in this experiment were t_0 and t_1 . For example, R_0 is FLV_0 , divided by the number of total number of virus particles (stained and unstained), C_0 , at time 0.

The mean specific activity, \bar{R} , is then calculated as

$$\bar{R} = \left(\frac{R_0}{k \times t}\right) \times (1 - e^{-kt}) \quad (2)$$

The viral decay or removal rate, D_v , is calculated as

$$D_v = \frac{(FLV_0 - FLV_t)}{(\bar{R} \times t)} \quad (3)$$

where FLV_0 and FLV_t are the concentrations of FLV at t_0 and at time t , respectively.

The viral production rate, P_v , is calculated as

$$P_v = \frac{\ln\left(\frac{R_0}{R_t}\right)}{\ln\left(\frac{C_0}{C_t}\right) \times t} \times (C_0 - C_t) \quad (4)$$

where C_0 and C_t are the concentrations of virus particles at t_0 and time t , respectively.

If the virus abundance does not change over time, then the removal rate is equal to the production rate (and the equation is not used). For each experiment, initial rates (using the first two time points, t_0 and t_1) and overall rates (using the entire time course) of production and decay are calculated. Initial rates of decay/production are closest to in situ rates, as all of the experiments can be started at dusk and held under ambient natural conditions. Overall rates represent decay/production under natural conditions for ~12 h, but samples held in the dark the following morning should not be exposed to natural sunlight.

Estimates of viral-induced bacterial mortality can be calculated using overall rates of virus production, mean viral abundance, mean bacterial abundance and growth rates, and either an empirically measured or estimated burst size. Briefly, virus

production rates are divided by the estimated burst size (we used a range from 20 to 50) to determine the number of bacterial cells killed $L^{-1} day^{-1}$. The researcher can divide the number of bacterial cells killed $L^{-1} day^{-1}$ by the rate of bacterial production in cells $L^{-1} day^{-1}$, to determine the portion of the bacterial community killed due to viral lysis. All of the provided calculations assume steady state.

Assessment

Creation of FLVPs—The optimization of the staining protocol using SYBR Green I resulted in high-quality, monodispersed FLVPs after very short incubation times (~15 min). The use of distilled water, versus seawater, during preparations seemed essential in obtaining particles with the highest fluorescent yield. This comparison was done because (1) the cationic interference of some fluorescent dyes had been reported (Hennes and Suttle 1995) and (2) some marine viruses are unstable in distilled water owing to osmotic stress (Zachary 1976, Faruque et al. 2000). In the case of SYBR Green I, Noble and Fuhrman (1998) reported that the dye was not affected by seawater during the preparation of slides for epifluorescence microscopy on natural populations. Our trials suggest that interference does take place in the case of some pure virus stocks, however—effects that would not be seen in mixed, natural populations—which may be related to the nature of the particles themselves. Regarding long-term storage, only a minimal loss in stain quality was observed in the FLVP stock maintained at 4°C over 8 months. The effects of storage at -20/-80°C were not investigated, but we assume that FLVPs should be stable if they are made from a virus stock which itself is known to be stable during cryopreservation.

Evaluating the FLVP assay—The tagging protocol from Hennes et al. (1995) was modified for SYBR Green I and simplified, in part due to the more amenable characteristics of SYBR Green I compared to the original YOYO-1 and POPO-1 dyes. The high-quality FLVPs, at a high MOI, created clear halos around the host cells (Fig. 1), allowing easy identification and discrimination in mixed prokaryotic populations. The strong halos were an indication that the host-cell receptor for the PWH3a-P1 virus is in high concentration on the cell surface and is evenly distributed. During the course of experimentation, the observed receptor pattern did not change, regardless of the physiological status of the host. Nor were there any cells showing stain having penetrated into the cells (halos becoming diffuse, whole-cell staining), indicating that the FLVPs remained non-infectious (no DNA injection) and that there was no leakage of the stain out of the FLVPs into the cells. This is consistent with observations from other research groups, who have found that FLVPs made from a variety of phages remain non-infectious and the DNA stain is not injected. Nonspecific binding of the FLVPs to other prokaryotes, eukaryotes, or detritus was not observed. The efficiency of the assay was tested by adding a known abundance of PWH3a cells, determined by independent acridine orange counts, to a



Fig. 1. *Vibrio alginolyticus* strain PWH3a tagged with FLVPs. FLVPs were created with SYBR Green I stain and demonstrate uniform attachment to the host cells.

natural population and subsequently recovering them using the assay. Cell counts were not significantly different (data not shown). The sensitivity of the assay depends on the standard statistical detection limit for epifluorescence microscopy, which is ~200 cells mL^{-1} (see Suttle and Fuhrman 2010, this volume). However, filtration of a larger sample volume when low abundances are suspected can increase this detection limit.

The viability of FLVP slides was investigated after storage at -20°C for varying lengths of time. For slides stored up to 54 days, the cell counts did not vary significantly (mean change 1.1%, $n = 6$). This observation is in accordance with Wen et al. (2004), who showed that freshly made epifluorescence slides maintain their viability for significant periods of time. Their study, however, did highlight significant problems when making slides from stored samples that had been fixed with aldehydes. Previous studies have reported incompatibilities of fluorescent stains with aldehydes, but SYBR Green I was purported to not suffer from this disadvantage (Hennes and Suttle 1995, Weinbauer and Suttle 1997, Noble and Fuhrman 1998). Even given this advantage, aldehyde fixatives cross-link membrane proteins, and their compatibility with the FLVP assay was doubtful owing to the assay's dependence on functional virus receptors on the cell surface. We therefore examined the effects of glutaraldehyde fixation on tagged cells because of the common use of this fixative with marine microbial samples. As expected, glutaraldehyde fixation inhibited adsorption of the FLVPs to the cells. In contrast, fixation after adsorption did not destroy the interaction between the virus and its receptor, nor did it impede enumeration. Finally, as generally observed by Wen et al. (2004) for marine virus samples, a significant drop in FLVP-tagged cells was observed when these were stored in glutaraldehyde before slide preparation. Losses of 13% to 33% ($n = 7$) of the original cell counts were recorded within hours of initial fixation. The relative intensities of the FLVP-tagged cells remained essentially constant (i.e., FLVPs stayed

attached), indicating that these drops in counts were actual losses of individual cells and probably not decay of FLVPs on the surface of the cells making them uncountable.

FLVP microcosm experiments—Two microcosm experiments were conducted to demonstrate the capability of the FLVP assay to resolve ecological interactions by following one species within natural communities. The first experiment involved the addition of the host strain PWH3a to a natural community under conditions where the former did not dominate the system (Fig. 2). PWH3a composed 75% of the total prokaryotic community at the outset, and high nutrient conditions (10% MLB) were used, as it was anticipated that these would favor the PWH3a strain given that *Vibrio* spp. are commonly copiotrophic. However, the incubation temperature of 20°C was not at its optimal, as this strain was originally isolated in the Gulf of Mexico and preferentially maintained at 30°C. Consequently, other prokaryotes in the community increased in concentration to dominate the system while PWH3a decreased slightly in concentration. Upon visual inspection of the microcosm, a bloom of organisms was apparent after the first couple of days. The color of the bloom was consistent with PWH3a, but was proven incorrect by the FLVP assay. Last, concentrated PWH3a-P1 virus was added to the microcosm shortly after 72 h, and a resulting disappearance of PWH3a was recorded. In the second microcosm, PWH3a was again added to a natural community (Fig. 3). This time, the concentration of PWH3a was altered so that it composed ~98% of the total community at the outset, and the incubation temperature was also raised to 30°C in a further effort to promote PWH3a dominance. As the FLVPs reveal, this dominance was achieved and maintained until PWH3a-P1 virus was again added to lyse the cells. Upon removal of PWH3a, other prokaryotes, presumably suppressed earlier, were then able to dominate the community 24 h later.

FLVP receptor titration experiment—Attachment to the host receptor is crucial in viral infection, and receptor quantity is sometimes tied to physiological status (differential receptor expression), such as for the maltoporin receptor of phage λ (Boos and Shuman 1998), or is sometimes simply a matter of cell surface dimensions, as in the case of fixed receptor components such as lipopolysaccharide for phage T4 (Thomassen et al. 2003). Theoretically, FLVPs could be used to roughly titer the number of receptor sites by testing for resistance to infection—host cells with enough attached FLVPs should have their receptor sites saturated, preventing adsorption of normal (unstained) infecting phage. We attempted to demonstrate this by pre-incubating PWH3a cells with two different MOIs of FLVPs, 1000 and 10, following an attempt to infect them by subsequently adding normal PWH3a-P1 virus. Infection of PWH3a coated with the lower MOI of FLVPs did not seem to be affected, as the cells were lysed at the same rate as the control cells (no FLVPs; Fig. 4). The PWH3a cells pre-incubated with the higher MOI of FLVPs, however, did resist infection. The abundance decreased slightly in the first 4 h after infection,

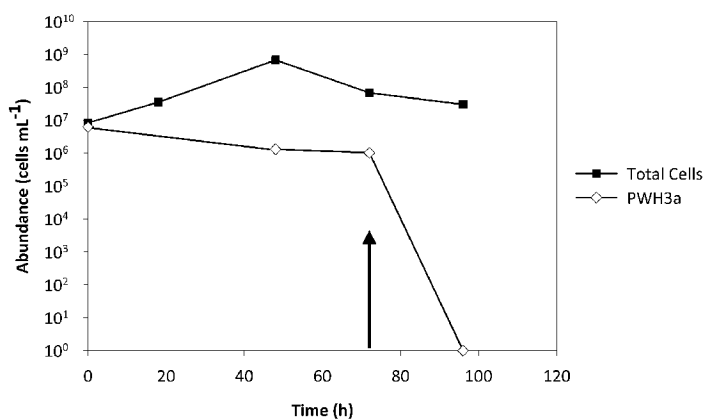


Fig. 2. Addition of *Vibrio alginolyticus* strain PWH3a to a natural community of prokaryotes, where the exogenous bacterium did not dominate the system. PWH3a counts and total prokaryote counts are represented, as well as the time point (~72 h) at which PWH3a-P1 virus was added (arrow). Error bars representing the range of duplicate samples are smaller than the width of the symbols.

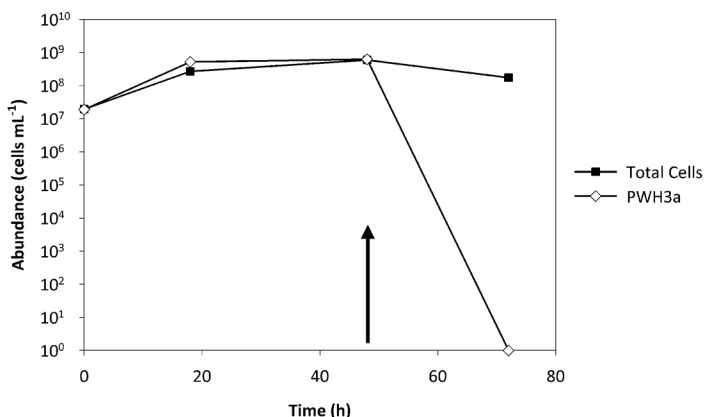


Fig. 3. Addition of *Vibrio alginolyticus* strain PWH3a to a natural community of prokaryotes, where the exogenous bacterium dominates the system. PWH3a counts and total prokaryote counts are represented, as well as the time point (49 h) at which PWH3a-P1 virus was added (arrow). Error bars representing the range of duplicate samples are smaller than the width of the symbols.

presumably as some less protected cells (with slightly less than 1000 FLVPs cell⁻¹) were successfully infected and lysed. After this time period, however, the PWH3a population growth paralleled the uninfected control. These observations show that FLVPs can act as inhibitors to slow down the kinetics of infection. Additionally, carefully adjusted MOIs of FLVPs may be useful in roughly titrating the relative amounts of viral receptor molecules on host cells.

FLVs as tracers—Since the late 1990s, several groups have reported the use of FLVs as tracers (e.g., Breitbart et al. 2004, Helton et al. 2005). In one study, the authors used FLVs to determine rates of virus production in hot spring environments in the Sierra Mountains of Central California. Breitbart et al. (2004) observed rates of phage production of 1×10^9 to

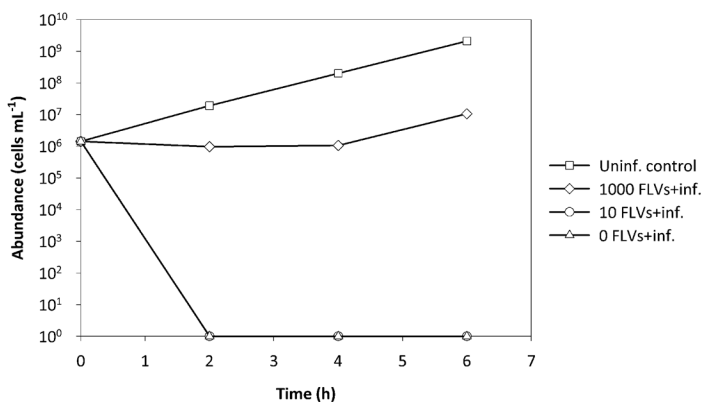


Fig. 4. Titration of *Vibrio alginolyticus* strain PWH3a phage receptors by pretreatment with FLVPs. PWH3a counts are represented for the four conditions, in order: uncoated (no FLVPs), uninfected control; PWH3a-P1-infected with 1000 FLVPs cell⁻¹ pre-coating; PWH3a-P1-infected with 10 FLVPs cell⁻¹ pre-coating (under triangles); and PWH3a-P1-infected with no pre-coating.

1.5×10^9 viruses L⁻¹ day⁻¹ at both sites. The turnover times observed in this study were similar to those measured in other near-shore marine and freshwater communities (Breitbart et al. 2004). The authors employed several alterations to the previously published FLV method by Noble and Fuhrman (2000). These alterations are noted in the described protocol here as possible alternative steps. Another study was conducted to compare the available incubation-based approaches for measuring virus production in estuaries (Helton et al. 2005). In this study, the authors determined that the FLV tracer assay overestimated virus production in highly eutrophic waters, since greater than 100% of the measured bacterial production would have been consumed through viral lysis, according to their measurements. There are a few reasons for the observed results in the published estuary study. First, estuarine samples are difficult to work with for FLV additions. After several attempts at FLV additions to other highly eutrophic estuarine environments in eastern North Carolina, we have observed a high likelihood for FLVs to attach to large abiotic particles, making accurate enumeration at time 0 particularly patchy and difficult to obtain. Second, it is difficult to enumerate the FLVs in such waters, given the high levels of detritus and cyanobacteria and high eubacterial abundances. Finally, it is highly possible that over time, FLV numbers are reduced due to irreversible binding to both abiotic and biotic (suspended solids and phytoplankton) particles, processes that have nothing to do with the intended measurement of viral production. We suggest that the FLV tracer assay was intended originally for use in oceanic or extreme (e.g., hot spring) environments, which is where the protocol was optimized. Dilution-based approaches are optimal for measuring virus production in estuarine environments, and this has been empirically shown in several estuarine locations (Helton et al. 2005; R. T. Noble, unpublished data).

Discussion

The FLVP assay has been demonstrated as a sensitive and efficient method for enumerating single prokaryotic species. The FLV tracer assay has been used in a range of different environments, and researchers that have used the method in extreme environments (hot springs) reported excellent success with this approach. Some of the possible ecological interactions that can be resolved with these assays are presented herein, as well as possible uses of FLVs as inhibitors of infection kinetics or as titrators of viral receptors. Traditional culture methods have been inefficient at these tasks, and alternative modern techniques, such as fluorescent in situ hybridization (FISH) with rRNA probes (Amman et al. 1995) or immunofluorescence (Middelboe et al. 1996), have more complex protocols and requirements. The FLV assay's sole requirements are (1) a virus for the host of interest (for probes); (2) sufficient receptor sites for the virus on the host cell surface; and (3) an adequate fluorochrome, such as one of the effective cyanine dyes optimized in this study. Given these facts, the FLV-based assays should be powerful tools in applied settings and in aquatic microbial ecology.

Comments and recommendations

The FLV assays described in this chapter should be relatively robust, and changing fluorescent dyes and/or virus-host systems will probably require only small changes, if any, to the protocols developed herein, possibly regarding the ratio of viruses to hosts and/or the adsorption times in the FLVP assay. FLVs have been successfully made from a variety of viruses and using a multitude of fluorochromes, even as integrated capsid fusions to fluorescent proteins (Tanji et al. 2004, Slootweg et al. 2006). The FLVP assay has thus far been limited to bacteriophages, however, and a potential future use of the assay would be to expand to eukaryotic viruses to track the dynamics of individual microalgae. Certain algal viruses, such as the *Heterosigma akashiwo* virus HaNIV (Lawrence et al. 2001), have burst sizes that should be adequate to generate the high titer stocks needed for FLVP preparation. Additionally, Hennes et al. (1995) showed that the autofluorescence of autotrophic cells (in this case *Synechococcus* cyanobacteria) did not interfere with the FLVP assay when fluorochromes were appropriately selected and that the viral receptor sites remained available for the assay, even though their expression may be tightly linked to light exposure. The protocols developed herein are stringent with regard to protection from light to minimize stain fading; however, they may be relaxed (using subdued light conditions or short expositions to full light) if phototrophs show a marked, and quick, loss of receptors when placed in dark conditions. We suspect that occurrence of this problem should be relatively rare, as incubation with the FLVPs is relatively short (15–30 min) and that many receptors, even for viruses of phototrophs, are probably fairly integral components of cell membranes (such as lipopolysaccharide) without rapid recycling

permitting immediate receptor deletion. However, it is still important to determine whether there are enough viral receptors or whether their distribution on the cell surface allows for adequate enumeration, along with whether they remain available at all times throughout the assay.

It is possible that the future of marine microbial ecology could include the concept of tagging virus concentrates with quantum dots. This approach has been conducted for human pathogenic viruses, such as HIV, and has been detailed in a variety of journal articles (e.g., Kampani et al. 2007). Quantum dots can be purchased commercially, can be linked to the viruses in a variety of ways, and should not suffer from the fading problems associated with the SYBR-based dyes used previously in FLV-type studies. It is possible that quantum dot-labeled viruses could be used as probes similar to those used in the FLVP assay and could facilitate enumeration and sorting of the tagged host cells through the use of equipment such as flow cytometry, the latter of which has only been used once (possible signal-to-noise ratio problems) with standard fluorochrome FLVPs (Goodridge et al. 1999).

Since development of the protocols in the late 1990s, several groups have made suggested improvements and modifications to conduct FLV tracer experiments under a range of conditions (i.e., at times when centrifuges are not available, or other equipment items cannot be used). The general approach and protocol presented can be modified in a number of ways to accomplish the goal of the researcher. The creation of the virus concentrate could be accomplished using a range of approaches not tested here (e.g., hollow fiber filtration, filter cartridges, filtration-elution). The original concept will benefit from further testing in a range of different aquatic environments. The FLV tracer assay was originally designed for use in oceanic environments and is likely an optimal approach for use in aquatic systems without high concentrations of detrital or particulate organic material. As presented by Helton et al. (2005) and noted in previous experiments conducted by the authors of this chapter, we suggest that the FLV assay may not be appropriate for highly eutrophic estuarine environments. Experiments conducted in the Neuse River Estuary, North Carolina, have basically shown that FLV tracer studies were not appropriate in waters with high levels of TSS (>50 mg/L) and high turbidity (R. T. Noble, unpublished data). However, we suggest that the FLV tracer approach is one of several methods that could be used in the coming years to better understand the roles and function of viruses in the global oceans.

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