

Obtaining targeted metagenomes from uncultivated environmental *Synechococcus* using flow cytometry-based viral tagging

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Abstract

Ocean viruses are abundant, ubiquitous, and play important roles in global biogeochemical cycles through mortality, horizontal gene transfer and manipulation of host metabolism. However, the ability to link viruses to their hosts in a high-throughput manner bottlenecks our ability to understand virus-host interactions in complex communities. Here, we present viral tagging (VT), a method that combines mixtures of isotope labeled host cells and fluorescent viruses with flow cytometry. In a single experiment, we can screen 10^7 uncultivated ocean viruses with a single strain of *Synechococcus*. These viruses can then be sequenced to quantitatively link objectively defined environmental viral populations, and their genomes, to their hosts.

Introduction

As ocean ecology attempts to advance from observations to predictions, modeling becomes of fundamental importance. While two decades of viral ecology research has focused on community level understanding through measuring viral abundance, production, decay, and frequency of infected cells (*reviewed by Ref¹*), little is known about the fundamental question of “who infects whom?”.

Cultivation-based methods can directly link a virus strain to its host; however, it is impossible to characterize millions of viruses per milliliter of seawater and thousands to 100s of thousands of “viral types” per sampling site² using traditional low-throughput cultivation methods. For example, Fluorescently Labeled Viruses (FLVs) have been used as probes to “tag” their host cells for examination under the microscope³⁻⁵, a method that is limited only by being low-throughput. Here, as illustrated in Fig 1, we expand upon the use of FLVs to tag host cells by (i) incorporating flow cytometry to enable high-throughput detection and sequencing of infected host cells, and (ii) isotope labeling host cells to minimize the bacterial DNA in the viral tagging metagenome.

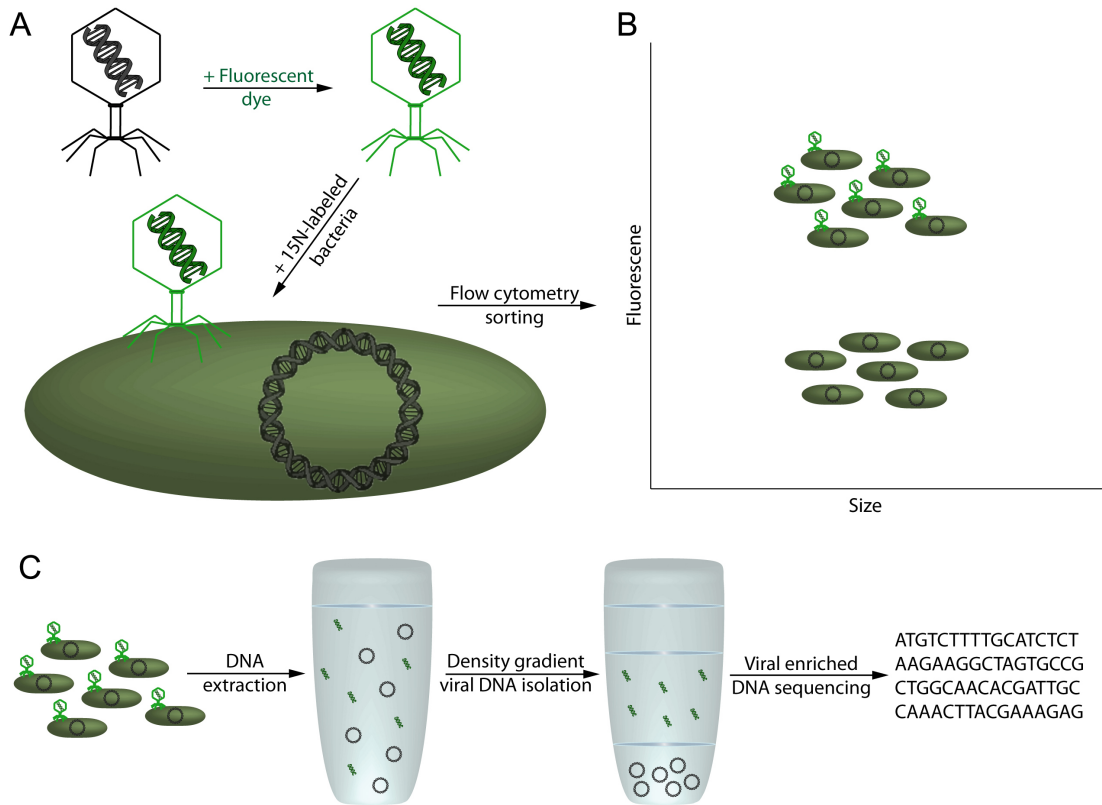


Figure 1. Overview for viral tagging (VT).

A. Environmental viruses are fluorescently-labeled green, then mixed with potential host bacteria which are labeled with heavy isotope, but flow cytometrically green-negative.

B. Flow cytometry data triggered on forward scatter for the fluorescently labeled virus and host bacteria co-incubated at desire VBR. Tagged cells infecting by viruses with extra fluorescence can be sorted out from non-infected cells.

C. DNA is extracted from the sorted, viral-tagged population. Isotopically light viral DNA is then separated from heavy host DNA using a density gradient for the downstream amplification and sequencing.

Reagents

1. ^{15}N ammonium chloride (Cambridge Isotope Laboratories, Inc. NLM-467-1).
2. Quant-iT Pico Green (Invitrogen P7589).
3. SYBR Gold (Invitrogen S11494)
4. TE buffer (10mM Tris, 1mM EDTA)
5. MTN buffer (0.6M NaCl, 0.1M Tris-Cl pH 7.5, 0.1M MgCl_2)
6. Bovine serum albumin (BSA)
7. Phosphate buffered saline (PBS)
8. Fluorescent polystyrene FLOW CheckTM microspheres (1 μm yellow-green beads; Polysciences Inc., PA, cat# 23517-10)

Equipment

1. Ultracentrifuge, we used a Beckman L70 ultracentrifuge with Beckman VTi 65 vertical rotor and 13x48 mm OptiSeal polyallomer tubes (4.9 ml capacity).
2. Appliskan plate reader (Thermo Electron, Vantaa, Finland).
3. Sonication bath, we used VWR Signature Ultrasonic cleaner B1500A-DTH.
4. Refrigerated centrifuge, we used Eppendorf 5417R.
5. Flow cytometer, we used iCyt Reflection flow cytometer (Sony Biotechnology) and a MoFlo™ XDP cytometer (Beckman Coulter).
6. Nanosep 10K centrifugal tubes (Pall OD010C33)

Procedure

Isotopic Labeling of Cyanobacteria and DNA Analysis

1. Prepare SNAX medium according to directions⁶, but use ¹⁵N ammonium chloride which will provide the heavy isotope. Grow cyanobacteria in the medium with heavy nitrogen and transfer at least 3 times before use.
2. Extract DNA from bacterial grown in heavy nitrogen using standard methods.
3. Quantify the DNA using Quant-iT Pico Green (Invitrogen #P7589).
4. Use at least 10 µg of DNA for density gradient centrifugation.
5. For density gradient centrifugation, a Beckman VTi 65 vertical rotor was used with 13x48 mm OptiSeal polyallomer tubes (4.9 ml capacity).
6. Mix the DNA with TE buffer (10mM Tris, 1mM EDTA, pH7.6) to a final volume of 0.9 ml.
7. Mix the DNA with 4ml of CsCl prepared in TE to a density of p1.8 (measure the density of the final solutions, they should be p1.7).
8. Dispense 4.9 ml of the DNA sample in CsCl into the OptiSeal tube and plug with the black caps.
9. Centrifuge at 44,000 rpm (=184,678.5 g) in a Beckman L70 or L80 ultracentrifuge for 48 hr at 18°C (Caution: do not centrifuge at lower temperatures as CsCl may precipitate out).
10. Collect 0.2-0.25 ml fractions and measure the density of each.
11. Calculate amount of DNA in each fraction using Quant-iT Pico Green (perform in duplicate) to determine the density of the fractions with DNA. Estimate the ratio of isotopic labeled cells.

Preparation of SYBR Gold working stock

1. Thaw 10,000x stock of SYBR Gold in the dark and vortex vigorously.
2. Centrifuge for 10 min at 3000 x g.
3. Dilute 1:100 (to 100x) with TE buffer and then filter through 0.02µm filter.
4. Aliquot into 50 or 100µl amounts and store frozen at -20°C.

SYBR-Gold Staining of Virus

1. Thaw prepared 100X SYBR Gold the dark, one time only (do not refreeze).
2. Vortex thawed SYBR Gold vigorously before using to stain viruses.

3. If using CsCl-purified virus, dialyze in modified MTN buffer.
4. Stain the viruses by adding prepared SYBR Gold to the viral suspension to a final concentration of 5x for a concentrated viral stock (equal to or greater than E+09) or 1x for less concentrated viral stock (<E+09). Perform these activities in lowered light as SYBR Gold will degrade upon exposure to light. The final volume should be 500µl.
5. Prepare a blank without virus to assess how well the excess SYBR Gold is washed away.
6. Vortex the sample for 10 sec on high to mix the dye and viruses.
7. Incubate 10 min in the dark at room temperature.
8. Heat at appropriate temperature for 10 min, using foil to cover the heat block to keep the sample in the dark. The staining temperature will depend on the host-virus system used, the infectivity of stained viruses in various temperature should be pretested.
9. Cool down the sample in the dark at room temperature for 10 min. Do not vortex the sample when it is still hot or you will disrupt the phage particles.

Wash Background Stain Away

1. You will need one Nanosep 10K centrifugal tube for each stained virus preparation plus one for the SYBR-Blank.
2. Prepare 0.02µm filtered 1% bovine serum albumin (BSA; equal to 10 mg/ml) in phosphate buffered saline (PBS); this should be filtered fresh before viral washing.
3. Wash Nanosep 10k tubes by adding 500µl MTN buffer, let stand 10 min, then spin until almost dry (5,000 x g, 10 min) and discard the flow-through.
4. Add 500µl 0.02µm filtered 1% BSA, let stand 1hr at room temperature and spin until almost dry (5,000 x g, 15-30 min); discard the flow-through.
5. Then wash with 500µl 0.02µm filtered MTN buffer.
6. Concentrate the 500µl stained and cooled virus preparations and the SYBR-Blank in the pre-treated Nanosep devices using 3,000 x g for 15-30 min, at 10°C to get to <50µl volume.
7. Wash the reduced volume of stained virus or SYBR-Blank with 500µl 0.02µm filtered MTN buffer and repeat spin as in step #6.
8. Repeat #7 for a total of 6 washes being sure to bring volume to <50µl each time.
9. Transfer the 50µl of washed viruses and SYBR-Blank from the Nanosep into fresh collection tubes (1.5 ml centrifuge tubes) and keep the collection tubes on ice.
10. Add 50µl 0.02µm filtered buffer to each Nanosep and sonicate for 3 min using the settings of 50W at 42 kHz; then pipet up and down on the filter carefully so as not to puncture the membrane and add to the appropriate stained virus or blank tubes and transport the collection tubes of washed viruses on ice.

Virus Tagging

1. Perform the tagging as soon as possible after staining and washing the virus.
2. Count the host cells and the SYBR-stained viruses to determine the amount of each to add. The virus-to-bacterium ratio (VBR) will depend on the host-virus system used, but in general will be 1 to 10. Incubate washed viruses with isotope labeled host cells for 60 minutes.

3. Prepare the flow cytometer for use and trigger on forward angle or side scatter.
4. Examine the 0.02 μ m filtered MTN buffer and set voltages so that noise is minimized.
5. Examine the host cells alone to set voltages to determine proper concentration to use and to determine where they are located on the plot of 520nm vs scatter. Use fluorescent polystyrene FLOW CheckTM microspheres (final concentration is 1:1 to host cells) as an internal standard for counting and sorting.
6. Mix the host cells with the SYBR-Blank, incubate dark for 10 min and mix the tube gently. Examine the mixture – the host cells should not increase in 520nm fluorescence.
7. Mix the host cells with the stained and washed virus at the appropriate VBR and examine after appropriate incubation times. If the stained viruses attach to the host cells, then the cells will show an increase in fluorescence at 520nm.
8. Sort and collect the viral-tagged cells, which are of increased fluorescence.
9. Sorted viral-tagged cells can be subjected to DNA extraction and separation of ¹⁵N-labeled “heavy” host DNA from non-labeled “light” viral DNA by CsCl density ultracentrifugation. See “Cesium Chloride Virus Purification and Dialysis” protocol⁷.
10. Light DNA can be linker amplified⁸ for sequencing by 454 Roche Titanium and Illumina HiSeq 2000. Please note that our linker ligation step of the library prep strongly selects against ssDNA and further, RNA viruses would not be sequenced in a DNA metagenome.

Troubleshooting

1. Increase in 520 nm fluorescence of the mixture of cells and the SYBR-Blank. The washing procedure for the viruses is probably not adequate.
2. No increase in 520 nm fluorescence of the mixture of cells and the strained and washed viruses. The washing procedure for the viruses might have failed and lost most of the viruses, count the number of viruses recovered from washing. Otherwise increase the VBR to add more viruses per cell.

Time Taken

The time required for isotope labeling of host cells is variable, for 3 inoculation of *Synechococcus* WH7803 are about 3-4 weeks. The viral wash steps usually take about 3hs. The sorting takes about 1hour for E+08 cells, depending on the number collected and the experience of the investigator. Thus the whole procedure can be accomplished within 4-5 weeks.

Anticipated Results

Following the viral tagging method and DNA isolation and extraction, resulting DNA can be sequenced and used for viral community analysis (see Figure 1).

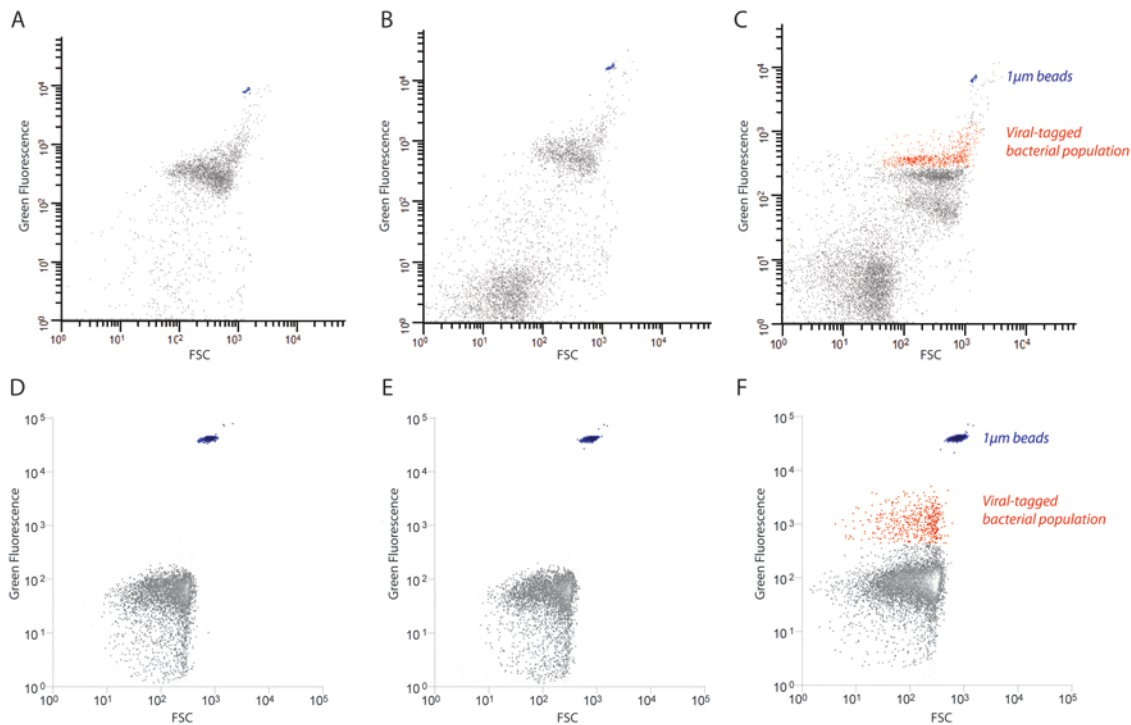


Figure 2. Reference flow cytometry charts.

A-C. Reflection data of viral-tagged samples.

A. Bacteria alone: 15N-Syn WH7803

B. No viruses staining control: 15N-Syn WH7803 + stained and washed buffer without adding viruses. A green negative and FSC negative noise population appeared in bottom left corner of plot, negative to bacterial primers.

C. Viral-tagging sample 1: 15N-Syn WH7803 + stained and washed viruses

D-F. MoFlo data of viral-tagged samples.

D. Bacteria alone: Syn WH7803

E. No viruses staining control: Syn WH7803 + stained and washed buffer without adding viruses.

F. Viral-tagging sample 1: Syn WH7803 + stained and washed viruses

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Associated Publications

- Deng, L., Ann Gregory, Suzan Yilmaz, Bonnie T. Poulos, Philip Hugenholtz and Matthew B. Sullivan. Contrasting Life Strategies of Viruses that Infect Photo- and Heterotrophic Bacteria, as Revealed by Viral Tagging. *mBio* **3**, 6 e00373-12 (2012).
- Deng, L., Ignacio-Espinoza, J.C., Gregory, A., Poulos, B.T., Weitz, J.S., Hugenholtz, P., & Sullivan, M.B. Viral tagging reveals discrete populations in *Synechococcus* viral genome sequence space. *in press*.