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

Li Deng, Bonnie T. Poulos, Matthew B. Sullivan

Abstract

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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.

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Guidelines

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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 Ref*[1]), 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 site[2] 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 microscope[3-5], 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.

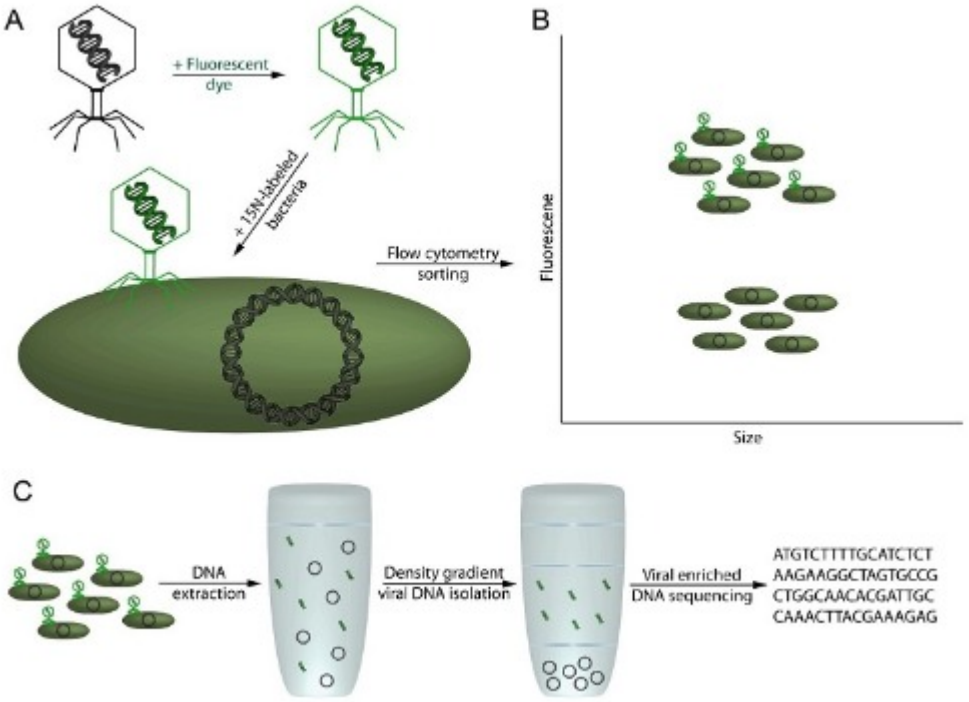

Figure 1. Overview for viral tagging (VT).
A. Environmental viruses are fluorescently-labeled green, then mixed with potential hostbacteria which are labeled with heavy isotope, but flow cytometrically green-negative.
B. Flow cytometry data triggered on forward scatter for the fluorescently labeled virus 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 MgCl2)
6. Bovine serum albumin (BSA)
7. Phosphate buffered saline (PBS)
8. Fluorescent polystyrene FLOW CheckTM microspheres (1µm yellow-green beads; Polysciences Inc., PA, cat# 23517-10)

Equipment

1. Ultracentrifuge, we used a Beckman L70 ultracentrifuge with Beckman VTi 65 vertical rotor

and13x48 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)

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 2).

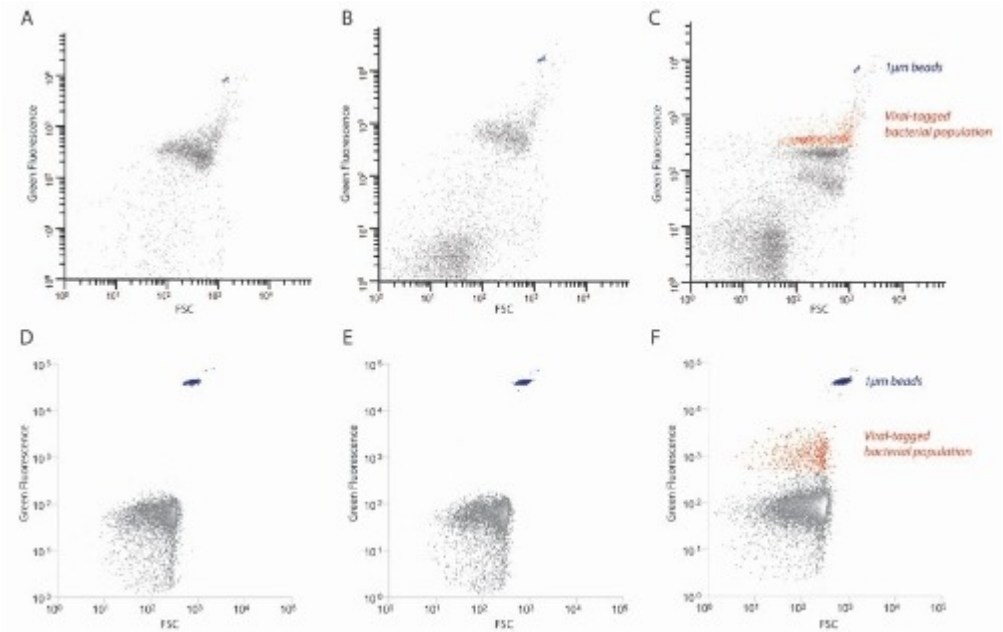

Figure 2. Reference flow cytometry charts.

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 withoutadding 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*.

Materials

Quant-iT™ PicoGreen® dsDNA Assay Kit P7589 by Life Technologies

Protocol

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 1.

Prepare SNAX medium according to directions[6], but use ^{15}N ammonium chloride which will provide the heavy isotope

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 2.

Grow cyanobacteria in the medium with heavy nitrogen and transfer at least 3 times before use

NOTES

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Do the 3 weekly transfers at 1:5 before any VT work, even when working with unlabeled cells. Cells grown in 24h constant light.

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But see step 12; typical Cyano maximum labeling is 75% of all cell DNA, and can take 5 weekly transfers to achieve.

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 3.

Extract DNA from bacterial grown in heavy nitrogen using standard methods

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 4.

Quantify the DNA using Quant-iT Pico Green (Invitrogen #P7589)

REAGENTS

Quant-iT™ PicoGreen® dsDNA Assay Kit P7589 by Life Technologies

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 5.

Use at least 10 µg of DNA for density gradient centrifugation

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 6.

For density gradient centrifugation, a Beckman VTi 65 vertical rotor was used with 13x48 mm OptiSeal polyallomer tubes (4.9 ml capacity)

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 7.

Mix the DNA with TE buffer (10mM Tris, 1mM EDTA, pH7.6) to a final volume of 0.9 ml

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 8.

Mix the DNA with 4ml of CsCl prepared in TE to a density of p1.8

NOTES

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Measure the density of the final solutions, they should be p1.7.

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 9.

Dispense 4.9 ml of the DNA sample in CsCl into the OptiSeal tube and plug with the black caps

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 10.
Centrifuge at 44,000 rpm (=184,678.5 g) in a Beckman L70 or L80 ultracentrifuge for 48 hr at 18°C

DURATION
48:00:00

NOTES
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Caution: do not centrifuge at lower temperatures as CsCl may precipitate out

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 11.
Collect 0.2-0.25 ml fractions and measure the density of each

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 12.
Calculate amount of DNA in each fraction using Quant-iT Pico Green (perform in duplicate) to determine the density of the fractions with DNA

REAGENTS
Quant-iT™ PicoGreen® dsDNA Assay Kit P7589 by Life Technologies

Isotopic Labeling of Cyanobacteria and DNA Analysis

Step 13.
Estimate the ratio of isotopic labeled cells

Preparation of SYBR Gold working stock

Step 14.
Thaw 10,000x stock of SYBR Gold in the dark and vortex vigorously

NOTES
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Check stock for orange particulates before diluting. Do not remove DMSO before diluting. Can freeze-thaw stock multiple times to make more dilutions.

Preparation of SYBR Gold working stock

Step 15.
Centrifuge for 10 min at 3000 x g

DURATION
00:10:00

Preparation of SYBR Gold working stock

Step 16.
Dilute 1:100 (to 100x) with TE buffer and then filter through 0.02µm filter

Preparation of SYBR Gold working stock

Step 17.
Aliquot into 50 or 100µl amounts and store frozen at -20°C

SYBR-Gold Staining of Virus

Step 18.
Thaw prepared 100X SYBR Gold the dark, one time only

NOTES
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Do not refreeze.

SYBR-Gold Staining of Virus

Step 19.

Vortex thawed SYBR Gold vigorously before using to stain viruses

SYBR-Gold Staining of Virus

Step 20.

If using CsCl-purified virus, dialyze in modified MTN buffer

NOTES

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If possible, avoid using CsCl-purified viruses. 0.2 um filter all phage samples day of use (recommend to be done twice). Concentrate samples if viruses are below 10^8 or 10^9/ml; use MTE buffer when concentrating.

SYBR-Gold Staining of Virus

Step 21.

Stain the viruses by adding prepared SYBR Gold to the viral suspension to a final concentration of 5 to 10x for a concentrated viral stock (equal to or greater than E+09) **or** 1x for less concentrated viral stock (<E+09)

NOTES

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Perform these activities in lowered light as SYBR Gold will degrade upon exposure to light. The final volume should be 500µl.

SYBR-Gold Staining of Virus

Step 22.

Prepare a blank without virus to assess how well the excess SYBR Gold is washed away

SYBR-Gold Staining of Virus

Step 23.

Vortex the sample for 10 sec on high to mix the dye and viruses

DURATION

00:00:10

SYBR-Gold Staining of Virus

Step 24.

Incubate 10 min in the dark at room temperature

DURATION

00:10:00

SYBR-Gold Staining of Virus

Step 25.

Heat at 46°C for 15 minutes inside hybridization oven (not heat block), inverting tubes every 5 minutes, using foil to keep the sample in the dark

DURATION

00:15:00

NOTES

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The staining temperature will depend on the host- virus system used, the infectivity of stained viruses in various temperature should be pretested (room temperature to 80°C).

SYBR-Gold Staining of Virus

Step 26.

Cool down the sample in the dark at room temperature for 10 min

DURATION

00:10:00

NOTES

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Do not vortex the sample when it is still hot or you will disrupt the phage particles.

Wash Background Stain Away

Step 27.

Prepare 0.02µm filtered 1% bovine serum albumin (BSA; equal to 10 mg/ml) in phosphate buffered saline (PBS)

NOTES

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This should be filtered fresh before viral washing.

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You will need one Nanosep 10K centrifugal tube for each stained virus preparation plus one for the SYBR-Blank.

Wash Background Stain Away

Step 28.

Wash Nanosep 10k tubes by adding 500µl MTN buffer, let stand 10 min

DURATION

00:10:00

Wash Background Stain Away

Step 29.

Spin until almost dry (5,000 x g, 10 min)

DURATION

00:10:00

Wash Background Stain Away

Step 30.

Discard the flow-through

Wash Background Stain Away

Step 31.

Add 500µl 0.02µm filtered 1% BSA, let stand 1hr at room temperature

DURATION

01:00:00

Wash Background Stain Away

Step 32.

Spin until almost dry (5,000 x g, 15-30 min)

DURATION

00:30:00

Wash Background Stain Away

Step 33.

Discard the flow-through

Wash Background Stain Away

Step 34.

Wash with 500µl 0.02µm filtered MTN buffer

Wash Background Stain Away

Step 35.

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

DURATION

00:30:00

Wash Background Stain Away

Step 36.

Wash the reduced volume of stained virus or SYBR-Blank with 500µl 0.02µm filtered MTN buffer

Wash Background Stain Away

Step 37.

Repeat spin as in step #36

DURATION

00:30:00

Wash Background Stain Away

Step 38.

Repeat #37&38 for a total of 6 washes being sure to bring volume to <50µl each time

Wash Background Stain Away

Step 39.

Transfer the 50µl of washed viruses and SYBR-Blank from the Nanosep into fresh collection tubes

NOTES

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1.5 ml centrifuge tubes.

Wash Background Stain Away

Step 40.

Keep the collection tubes on ice

Wash Background Stain Away

Step 41.

Add 50µl 0.02µm filtered buffer to each Nanosep and sonicate for 3 min using the settings of 50W at 42 kHz

DURATION

00:03:00

Wash Background Stain Away

Step 42.

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

Step 43.

Count the host cells and the SYBR-stained viruses to determine the amount of each to add

NOTES

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The virus-to-bacterium ratio (VBR) will depend on the host-virus system used, but in general will be 1 to 10.

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Perform the tagging as soon as possible after staining and washing the virus, no later than 10 hours after washing was done.

Virus Tagging

Step 44.

Use at least 2 ml of cells at 10^6 cells/ml, mix with appropriate concentration of virus particles and

incubate for 10-20 minutes to start detecting VT signal

DURATION
00:20:00

NOTES
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Up to 60 minutes may be required.

Virus Tagging

Step 45.
Prepare the flow cytometer for use and trigger on forward angle or side scatter

Virus Tagging

Step 46.
Examine the 0.02µm filtered MTN buffer and set voltages so that noise is minimized.

Virus Tagging

Step 47.
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

Virus Tagging

Step 48.
Use fluorescent polystyrene FLOW Check™ microspheres (final concentration is 1:1 to host cells) as an internal standard for counting and sorting

Virus Tagging

Step 49.
Mix the host cells with the SYBR-Blank, for 10 min and mix the tube gently

DURATION
00:10:00

NOTES
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Examine the mixture – the host cells should not increase in 520nm fluorescence.

Virus Tagging

Step 50.
Mix the host cells with the stained and washed virus at the appropriate VBR and examine after appropriate incubation times

NOTES
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If the stained viruses attach to the host cells, then the cells will show an increase in fluorescence at 520nm.

Virus Tagging

Step 51.
Sort and collect the viral-tagged cells, which are of increased fluorescence

Virus Tagging

Step 52.
Trigger on forward scatter to only detect cell-sized particles

NOTES
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Some flow cytometers will use side scatter for the same purpose.

Virus Tagging

Step 53.

Sorted viral-tagged cells can be subjected to DNA extraction and separation of ^{15}N-labeled “heavy” host DNA from non-labeled “light” viral DNA by CsCl density ultracentrifugation (See "Cesium Chloride Virus Purification and Dialysis")

Virus Tagging

Step 54.

Light DNA can be linker amplified[8] for sequencing by 454 Roche Titanium and Illumina HiSeq 2000

NOTES

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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.