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Short communication

Isolation of the phycodnavirus PBCV-1 by biological laser printing

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ABSTRACT

The *Phycodnaviridae* family of viruses is diverse genetically but similar morphologically. These viruses infect eukaryotic algal hosts from both fresh and marine waters, and are an important component of aqueous environments. They play important roles in the dynamics of algal blooms, nutrient cycling, algal community structure, and possibly gene transfer between organisms. As such, it is important to identify new viruses within the *Phycodnaviridae* family. Biological laser printing (BioLP) was used to isolate single virus particles from solution. BioLP prints droplets containing a single virus particle directly onto a host medium, thereby enabling viruses to be isolated from unmodified samples. This manuscript demonstrates how BioLP can be used as a single-step method to separate and possibly identify viruses from complex environmental specimens.

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Members and prospective members of the family *Phycodnaviridae* constitute a diverse genetically, but similar morphologically, group of viruses with eukaryotic algal hosts from both fresh and marine waters (Dunigan et al., 2006). The family derives its name from two distinguishing characteristics: (i) "phyco," from their algal hosts, and (ii) "dna," because all of these viruses have dsDNA genomes (160–560 kb) (Wilson et al., 2005). The phycodnaviruses are among the virioplankton recently recognized as important ecological elements in aqueous environments (Chen et al., 1996). More than 50% of the carbon dioxide fixed on the planet is by phytoplankton (Behrenfeld et al., 2006), including cyanobacteria and eukaryotic microalgae. Therefore, phycodnaviruses play a significant ecological role in the life cycle of their algal hosts.

Accumulating genetic evidence indicates that phycodnaviruses, along with poxviruses, iridoviruses, African swine fever virus (ASFV), and the recently discovered Mimivirus (Raoult et al., 2004), have a common evolutionary ancestor, perhaps arising at the point of eukaryogenesis approximately 2.0–2.7 billion years ago (Brocks et al., 1999; Feng et al., 1997; Glansdorff, 2000; Han and Runnegar, 1992). All of these viruses share 9 gene products, and 33 additional gene products are present in at least 2 of these 5 viral families (Iyer et al., 2001, 2006; Raoult et al., 2004). Collectively, these viruses along with the other asfariviruses and coccolithoviruses are referred to as Nucleo-Cytoplasmic Large DNA Viruses (NCLDVs) (Iyer et al., 2001).

New aquatic viruses, including the phycodnaviruses, are continually being discovered as more environmental samples are examined. Ongoing metagenomic studies indicate a greater viral diversity than could have been imagined just a few years ago (Hambly and Suttle, 2005; Wommack and Colwell, 2000). The ease with which new viruses are found suggests that aquatic viruses may represent the greatest source of uncharacterized genetic diversity on Earth (Hambly and Suttle, 2005). Appreciation for this diversity is limited, in part, because algal viruses have only been identified for about 0.1% of the ~40,000 known eukaryotic algal species (Guiry and Rindi, 2005). Oddly, it is possible that both biological and genetic diversity are the unifying themes of the phycodnaviruses.

Phycodnaviruses are large icosahedrons (mean diameter of 160 ± 60 nm) and, where known, have an internal membrane that is required for infection. Phylogenetic analyses of the δ-DNA polymerases from the phycodnaviruses indicate that they are more closely related to each other than to other dsDNA viruses, and that they form a monophyletic group, consistent with a common ancestor (Wilson et al., 2005). The viruses fall into six clades, which correlate with their hosts, and each has been given genus status. Often the genera can be distinguished by additional properties, including lytic versus lysogenic life styles, or linear versus circular genomes (Wilson et al., 2005). Members of the genus *Chlorovirus* (chlorella viruses) infect fresh water algae, whereas members of the

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other five genera (*Coccolithovirus*, *Phaeovirus*, *Prasinovirus*, *Prymnesiovirus*, and *Raphidovirus*) infect marine algae.

The chlorella viruses are relatively new to virologists but are the best-studied phycodnaviridae system since their initial discovery in 1981 (Van Etten et al., 1981). However, their discovery is contributing scientific and economic benefits despite our limited knowledge. For example, chlorella viruses are sources of new and unexpected genes (Fitzgerald et al., 2007a,b,c). These genes not only encode commercially important enzymes such as DNA restriction endonucleases, but many viral-encoded enzymes are the smallest member of their class. Consequently, these proteins often serve as ideal biochemical models for mechanistic and structural studies. For this reason and others, a new method was developed by which to isolate individual viral particles from aquatic samples that form plaques on algal host cell lawns. *Paramecium bursaria* chlorella virus 1 (PBCV-1) was used for these studies as it is the prototype chlorella virus.

For these experiments, the viral host *Chlorella* sp. NC64A was grown on MBBM medium as described previously (Van Etten et al., 1983; Van Etten et al., 1981). The cells were sub-cultured into fresh MBBM at a low density and grown to a concentration of 1.0–2.0 $\times 10^7$ cells/mL. The cells were then concentrated to 4.0 $\times 10^8$ cells/mL by centrifugation for 5 min at 5000 rpm using a Sorvall rotor at 4 °C, and the pellet was suspended in fresh MBBM. The concentrated *Chlorella* NC64A suspension (300 μL) was added to a 30 mm × 100 mm tube containing 5 mL of MBBM soft agar (0.75% MBBM agar) and mixed briefly by rolling the tube between the palms of the hands. The contents were poured onto a solidified 25 mL MBBM agar (1.5% agar) plate.

Cell printing is defined as the ability to drop-and-place living cells onto a surface with micron-scale precision (Ringeisen et al., 2006). There are several types of cell printers described in the literature ranging from jet-based to extrusion-based techniques (Ringeisen et al., 2006). In general, jet-based techniques are more precise than extrusion-based methods, and typically deposit low-volume droplets (0.5–1000 pL) in rapid succession (100–10,000/s) (Barron et al., 2005a; Ringeisen et al., 2002; Roth et al., 2004; Xu et al., 2006). Due to the low volume of each printed droplet, biological laser printing, or BioLP, is able to pattern single cells onto different surfaces with micron-scale resolution (Barron et al., 2005b; Othon et al., 2008; Xu et al., 2004). In addition, previous studies have determined that cells deposited by BioLP are unharmed as well (Barron et al., 2004b; Chen et al., 2006; Hopp et al., 2005; Ringeisen et al., 2001, 2004).

In this work, BioLP was used to isolate single virus particles from a concentrated solution. The BioLP apparatus, configured to print living cells from liquid culture, was described previously (Barron et al., 2004a,b) and a detailed description of how it was used for virus printing is described below. The concentrated virus suspension (2 μL) was spread evenly onto a 1-cm^2 quartz slide coated with

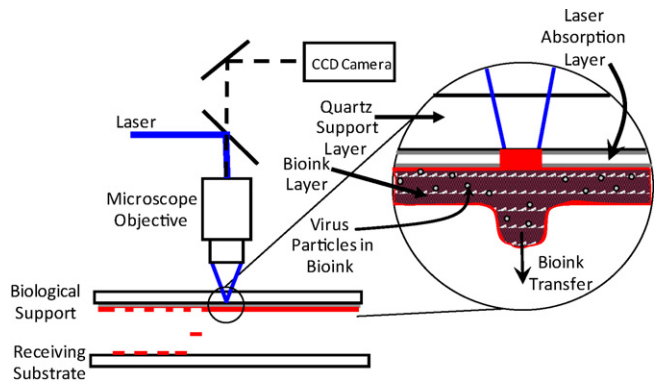

Fig. 1. Biological laser printing (BioLP) schematic. The support is comprised of three layers: a transparent support, an energy conversion layer (gray) and the liquid cell culture (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

a layer of 85-nm thick titanium dioxide. This slide was inverted and placed into the BioLP apparatus as shown in Fig. 1. A quadrupled UV Nd:YAG laser pulse (266 nm, 2 ns) was directed through a microscope objective and focused at the quartz/titanium dioxide interface of the biological support. This biological support is comprised of three layers: a transparent quartz slide, a thin laser absorption coating (an 85 nm thick TiO_2 layer), and a thicker biological fluid layer ("bioink" spread to ~10 μm thick). The light energy from the laser pulse was converted into heat energy at the quartz/TiO_2 layer, and the heat vaporized a portion of the virus culture medium (bioink) in direct contact with the metal oxide layer (Barron et al., 2004b). High speed images of this energy transfer process have show that a liquid jet is forced away from the quartz slide (Barron et al., 2005a). The quartz slide and receiving substrate were computer controlled and could be moved independently, such that with successive laser pulses, patterns could be directly written drop-by-drop onto the receiving substrate. For this study, a 300 spot array (~50 μm diameter droplets spaced 5 mm apart so that single plaques could form without merging with neighbors) was formed onto agar plates positioned directly below the quartz slide. The slide/agar plate distance was held at 0.5 mm throughout the duration of printing. The quadrupled UV Nd:YAG laser pulse used in this study has a laser frequency of 10 Hz, thus depositing 10 droplets per second onto the agar plates. However, it should be noted that a high-throughput laser printing device equipped with an IR pulsed laser has been shown to fire between 1 and 100 kHz equating to a possibility of printing 100,000 droplets per second (Guillemot et al., 2009).

Arrays of virus particles were printed onto the *Chlorella* NC64A lawn and were allowed to grow in continuous light at 25 °C. At low virus particle concentrations (10^8 pfu/mL), most printed spots

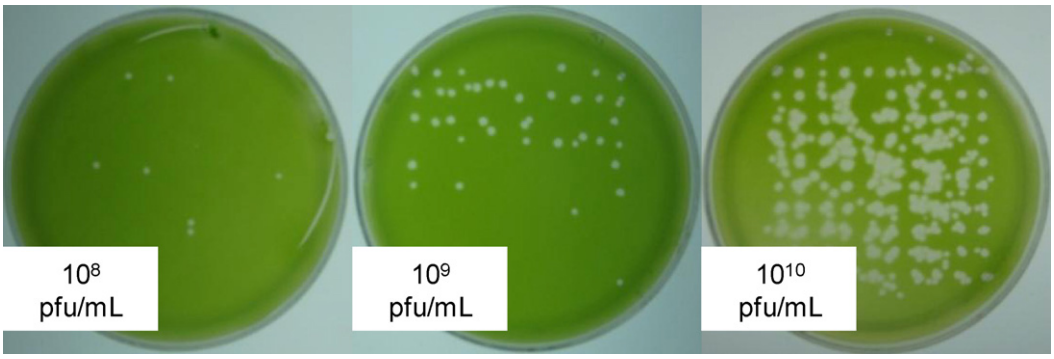

Fig. 2. Biological laser printing (BioLP) of chlorella virus PBCV-1 onto its host lawn of *Chlorella* NC64A.

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contained either one or zero plaque-forming virus particles as evidenced by the presence or absence of a circular plaque, respectively (Fig. 2). Higher concentrations of virus particles (10^{10} pfu/mL) yielded arrays with multiple plaques (i.e. multiple virus particles) printed per spot.

This is the first report showing the ability to print chlorella virus particles onto a host and opens the possibility to use this technique for other virus systems. There are several applications that could arise from such a technique. For example, one could identify new viruses belonging to the *Phycodnaviridae* family by printing environmental water samples onto different algal hosts to quickly isolate single virus particles. Established analytical methods that compare DNA polymerase (Clasen and Suttle, 2009) or major capsid sequence fragments (Larsen et al., 2008) could then be used to specifically identify the virus. As with bacterial printing, this method could lead to industrial or medical applications, where viruses could be printed for use as biological sensors, or human samples could be printed for rapid identification of infectious agents. In addition to the mentioned applications, this technique allows for the possibility of isolating new virus particles which would not be identified by using a standard plaque assay. With the advancement of high-throughput laser printing technology, one can print ~100,000 droplets per second (Guillemot et al., 2009). Therefore, with the ability to print multiple drops, one would be able to isolate low-titer virus particles. Furthermore, with the use of a 3456-well microtiter plate, one could quickly isolate over 3000 virus particles within milliseconds. This technique not only allows for rapid screening of environmental samples for the discovery of new virus particles but saves on reagent costs and sample scarcity.

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