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Fake virus particles generated by fluorescence microscopy

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Many laboratories are actively studying the abundance and roles of viruses in natural ecosystems. In these studies, the presence and number of viral particles is usually determined using fluorescent dyes. However, DNA associated with membrane-derived vesicles (MVs), gene transfer agents (GTAs), or cell debris can produce fluorescent dots that can be confused with viral particles. We suspect that fluorescence counting can lead to overestimation of virus numbers and even suggest the presence of viruses when there are none. Future studies in environmental virology should acknowledge this point and consider how to bypass this problem. Besides trying to improve discrimination between virions and MVs, we suggest adopting less holistic approaches, focusing on the detection of known virus groups and the isolation of new viruses from a broader range of hosts.

Finding viruses in the environment

In recent years, the interest in viruses as an integral part of the biosphere has been growing and several authors have proposed that viruses have played a much more active role in shaping biological evolution than it was previously believed [1–5]. In particular, ecological studies have suggested that viral particles (or more precisely virus-like particles, see below) are the most abundant biological entities on our planet [2,6,7]. Many laboratories have been actively studying the roles of viruses in regulating population dynamics and geochemical cycles in a variety of ecosystems, including humans. Viral ecology is thus becoming a thriving new branch of microbial ecology, itself an expanding discipline that attempts to combine traditional (cultivation) and molecular approaches such as shotgun metagenomics.

The number of viral particles in environmental studies is usually assessed by fluorescent staining, with SYBR Green being the most common dye used to label viral nucleic acids [8,9]. Fluorescent dots that are supposed to represent viral particles are visualized by epifluorescence microscopy (EFM) or counted by flow cytometry. Metagenomic analyses focusing on viromes (see Glossary) also rely on EFM as the method of choice to validate the presence of viruses and the absence of cellular contamination in environmental samples before DNA extraction and sequencing [10,11]. EFM, which is fast, easy, and inexpensive, has completely replaced the classical approaches of virus enumeration by plaque assay or by transmission electron microscopy (TEM) [12–14]. Theoretically, the use of EFM provides the possibility to count viruses that cannot be cultivated in laboratory conditions, and to discriminate between virions containing nucleic acids and nucleic acid-free virus-like particles. Contaminating free DNA is usually removed by DNase treatment [11].

The use of EFM was indeed a breakthrough in viral ecology and generated numerous publications, often in high profile journals [15]. However, the exclusive use of fluorescence staining in counting viral particles also raises several problems. A well-recognized one is that EFM is not efficient in detecting RNA or single-stranded DNA viruses [16]. This is an important issue because virome analyses have shown that these viruses are much more abundant in natural environments than previously expected [17,18]. We would like to focus here on another, less frequently

Glossary

Extracellular DNA: DNA present in the environment. This DNA can be attached to biological or mineral particles and can account for a large proportion (up to 70%) of all DNA present in a given environment.
Gene transfer agents (GTAs): particles resembling virions of tailed phages capable of encapsidation and transport of fragments of cellular DNA.
Kill the winner hypothesis: predicts that increase in the abundance of a specific host triggers significant increases in viral infection and subsequent lysis of this particular species, thereby controlling its abundance.
Membrane vesicles (MVs): globular vesicles (50–200 nm in diameter) produced by budding of the outer membrane in proteobacteria or the cytoplasmic membrane in archaea. They can fuse with recipient cells and carry cellular or plasmid DNA. Membrane vesicles have morphologies very similar to those of some spherical tailless viruses.
Microfluidic technology: enables control and manipulation of the behavior of fluids in microstructures. This technology has been successfully adapted for various single-cell studies.
Virus-like particle: general term that *a priori* covers true virions, GTAs, and MVs. Unfortunately, it is also often used as a synonym to virion or viral particle.
Virocells: virus-infected cells actively producing virions. Unlike regular cells, virocells cannot further divide. The virocell concept posits that infected cells are the organismal form of the virus.
Viromes: the complete set of viral genomes present in a given environment.

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discussed problem, i.e., to which extent a fluorescent dot in EFM studies really corresponds to a viral particle? In most studies, viral ecologists consider that the equivalence between the fluorescence count and the number of viral particles is obvious and does not need further elaboration. However, several recent observations suggest that this might not necessarily be the case.

Challenges to considering fluorescent dots as viruses

A recent paper in *Science* revealed that GTAs are abundant in the ocean [19]. In this environment, known to be rich in 'viral particles', GTAs mediate gene transfer at frequencies much higher than previously suspected. GTAs correspond to random pieces of cellular DNA packaged into capsids resembling typical tailed phages (Caudovirales) [7,20]. They are not viruses because proteins of the GTA 'coat particles' are usually not encoded by the random cellular DNA present inside the GTA, but by genes localized on the chromosome of the GTA-producing cell [20]. Because GTAs contain nucleic acids, they should cause false positive signals in EFM or flow cytometry studies designed to determine the number of viruses in natural environments. Unfortunately, to our knowledge, this has never been verified.

The problem of fake viruses in environmental virology is certainly not limited to GTAs. Indeed, it has been shown a few years ago that MVs produced by some hyperthermophilic archaea (*Thermococcus* and *Pyrococcus)* form fluorescent spots in EFM, even after DNase treatment [21] (Figure 1a,b). An EFM positive signal was observed after staining both culture supernatants and purified MVs. These MVs are 50–100 nm in diameter and some of them strikingly resembled virions produced by archaeal viruses [21]. They also resembled putative viruses previously observed in an ecological study of terrestrial hot springs (Figure 4 in [22]) but also in raw sewage (Figure 2C in [23]). MVs from *Thermococcus* and *Pyrococcus species* are associated to DNA, explaining why they stain positive in EFM analysis. However, DNA extracted from MV preparations purified by cesium chloride gradient was shown to represent fragments of cellular DNA [21]. Importantly, these MVs produced by hyperthermophilic archaea can protect DNA against thermodegradation at high temperature [21] and might play an important role in environmental gene transfers. Indeed, MVs can incorporate both endogenous and exogenous plasmids and transfer them between cells [24,25].

Although MVs have been too often considered as marginal cellular 'dust' by microbiologists, it is now more and more recognized that production of MVs is a universal and very important feature of cellular life [26–28]. Notably, Proteobacteria, which are abundant in most natural environments, produce MVs that can also package cellular DNA and protect it from digestion by DNases [29]. Consequently, as demonstrated for archaea, bacterial MVs also are likely to stain EFM positive. This indeed appears to be the case; when lysates from various bacterial species (*Synechococcus, Roseobacter, Roseovarius*, *Sulfitobacter*, and *Legionella*) that gave a positive EFM signal after DNase treatment were re-analyzed by TEM to confirm the presence of bacteriophages, genuine viral particles were found to be absent (for examples, see [30,31]). In several cases, no bacteriophages could be detected in samples that were estimated by EFM to contain up to 10^9 viral particles/ml. Importantly, all these lysates contained MVs of 30–50 nm in diameter of apparent bacterial origin

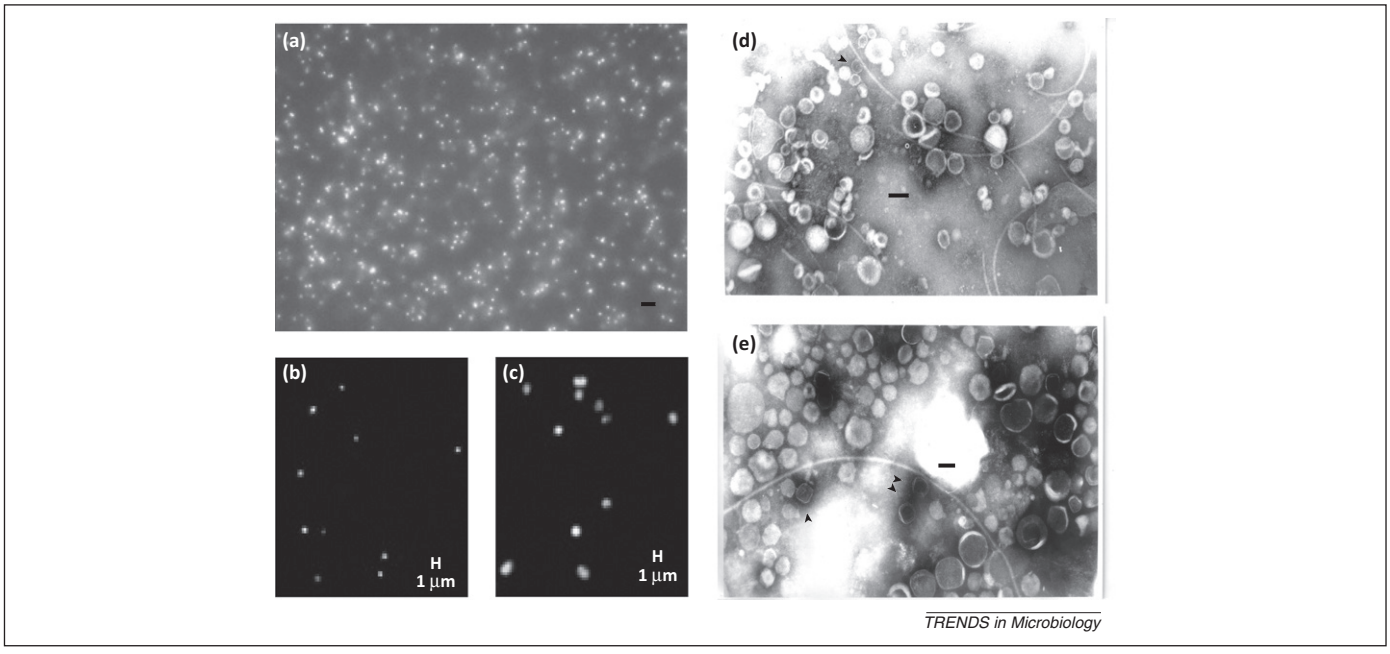

Figure 1. Microscopy of membrane vesicles and viruses. Epifluorescence microscopy (EFM) of membrane vesicles **(a,b)** and cells **(c)** from Thermococcales. (a) Culture supernatant of *Pyrococcus abyssi* strain GE5 stained with SYBR Green. (b) Purified membrane vesicles and (c) cells from *Thermococcus gammatolerans* stained with Hoechst dye (Hoechst, Darmstadt, Germany). Growth of strains and preparation of vesicles were carried out as previously described [20]. Scale bars, 1 μm. **(d)** Transmission electron microscopy of EFM-positive lysate of *Legionella* sp. showing a large number of virus-sized membrane vesicles. **(e)** Non-induced *Pseudomonas aeruginosa* phage lysate showing vesicles and a flagellum. Arrowheads in (d) and (e) point to putative empty viral capsids. Scale bars, 100 nm. Samples were concentrated by centrifugation at 25 000 g for 1 h in a Beckman JE-21 centrifuge (Beckman, Palo Alto, CA) and a JA-18.1 fixed angle rotor. After two washings in buffer, sediments were stained with 2% phosphotungstate and examined in a Philips EM 300 electron microscope.