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An optimized method for counting viral particles using electron microscopy

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Viruses can infect all types of life forms, from animals and plants to microorganisms, including bacteria and archaea. When studying samples containing viruses, one confronts an unavoidable question of the quantitative determination of viral particles in the sample. One of the simplest and efficient approaches to quantitative determination of viral particles in preparation includes the use of electron microscopy; however, a high detection threshold is a significant limitation of this method (10^7 particles per ml). Usually, such sensitivity is insufficient and can result in error diagnosis. This study aims to develop a method making it possible to detect the number of viral particles more precisely and work with samples in which the concentration of particles is lower than 10^7/ml. The method includes a concentration of viral particles on the polyethersulfone membrane applied in centrifugal concentrators and subsequent calculation using an electron microscope. We selected env-pseudoviruses using a lentiviral system making it possible to obtain standardized samples of virus-like particles that are safer than a live virus. Suspension of viral particles (a volume of 20 ml) was placed into the centrifugal concentrator and centrifuged. After that, we took a membrane out of the centrifugal concentrator and evaluated the number of particles on the ultrathin section using an electron microscope. The number of viral particles on the whole surface of the filter (a square of 4 cm^2) was 4×10^7 virions, the initial concentration of pseudoviruses in the sample was 2×10^6 per 1 ml (4×10^7 particles per 20 ml). As a result, the developed method enables one to evade the major disadvantage of quantitative determination of viruses using electron microscopy regarding a high detection threshold (concentration of particles 10^7/ml). Furthermore, the centrifugal concentrator makes it possible to sequentially drift a considerable volume of the suspension through the filter resulting in enhancement of test sensitivity. The developed approach results in increased sensitivity, accuracy, and reproducibility of quantitative analysis of various samples containing animal, plant or human viruses using electron microscopy.

Key words: electron microscopy; pseudoviruses; concentrating; number of viral particles.

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Оптимизированный метод подсчета количества вирусных частиц с помощью электронной микроскопии

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Вирусы поражают все типы организмов, от растений и животных до бактерий и архей. При исследовании образцов, содержащих вирусы, неизбежно встает вопрос количественного определения вирусных частиц в пробе. Одна из наиболее простых и эффективных методик количественного определения вирусных частиц в препарате – использование электронной микроскопии, однако основным ограничением метода является относительно высокий предел обнаружения (10^7 частиц/мл). Часто такая чувствительность недостаточна и может приводить к ошибочной диагностике. Цель данной работы заключалась в разработке методики, позволяющей более точно оценивать количество вирусных частиц и работать с образцами, в которых концентрация ниже, чем 10^7 частиц/мл. Метод заключается в концентрировании вирусных частиц на мембране из полиэфирсульфона, применяемой в центрифужных концентраторах, с последующим подсчетом с помощью электронного микроскопа. В качестве модельного объекта были выбраны env-псевдовирусы, созданные с использованием лентивирусной системы, которая позволяет получать стандартизованные образцы вирусоподобных частиц. Суспензию вирусных частиц (объемом 20 мл) помещали в центрифужный концентратор и центрифугировали. Затем извлекали мембрану из концентратора и оценивали количество осажденных на мембране частиц с помощью электронного микроскопа, используя метод ультратонких срезов. Количество вирусных частиц на всей поверхности фильтра (площадь 4 $см^2$) составляло 4×10^7 вирионов, исходная концентрация псевдовирусов в образце – 2×10^6 на 1 мл (4×10^7 частиц/20 мл). Таким образом, предложенная

методика позволяет преодолеть основной недостаток количественного определения вирусов с помощью электронной микроскопии, связанный с относительно высоким пределом обнаружения (10^7 частиц/мл). Кроме того, центрифужный концентратор дает возможность последовательно прогнать через один и тот же фильтр значительные объемы суспензии, содержащей вирусы, что также может привести к повышению чувствительности метода. Предложенный подход позволяет повысить чувствительность, точность и воспроизводимость количественного анализа различных образцов, содержащих вирусы животных, растений и человека, с использованием электронной микроскопии.
Ключевые слова: электронная микроскопия; псевдовирусы; концентрирование; количество вирусных частиц.

Introduction

At current, the interest in viruses has increased tremendously. Viruses affect all types of organisms, from plants and animals to bacteria and archaea. According to recent release of the International Committee on Taxonomy of Viruses, nearly 5000 species of viruses are presently known, and new viruses are discovered every year.

Modern diagnostic is an important element in the system of protection against infectious diseases; it determines adequate preventative measures and efficacy of further therapy. Visual detection and identification of an infectious agent with microscopy is an explicitly positive outcome of diagnostics. Electron microscopy is used widely to study viruses (Goldsmith, Miller, 2009). Apart from diagnostic purposes, electron microscopy is also employed for structural studies of nanoparticles, such as artificial VLP (virus-like particles), created to construct vaccine and for genome studies. Along with identification of particular nano-dimensional objects, electron microscopy gives important information about their morphology, survival rate under an impact of various physical and chemical (including pharmacological) factors, and enables to determine their content in biological fluids.

The main obstacle for use of electron microscopy in studies of viral objects is insufficient concentration of particles in fluids for the purposes of detection. To apply the simplest and the most popular method – negative staining, the concentration of virions (or other nano-scale objects) should be no less than 10^7 particles/ml (Reid et al., 2002; Malenovska, 2013). Such level of sensitivity, however, is often insufficient and can result in error diagnosis. To increase sensitivity, concentrating nanoparticles by means of ultracentrifugation is broadly used. It helps increase concentration of the analyzed particles per unit volume approximately thousandfold. At the same time, such work would require expensive equipment and a lengthy period of sample preparation.

Our study presents an original method for more accurate counting of viral particles. We illustrate the method proposed using env-presudoviruses as an example.

Materials and methods

Production of pseudoviruses. Pseudoviruses were obtained and characterized according to an earlier described method (Ryzhikov at al., 2012) with some modifications. HEK293T/17 cells were seeded in the 5×10^5 cells/hole concentration on a 6-hole cultural plate and incubated in an CO_2-incubator at +37 °C in DMEM, containing 10 % fetal bovine serum, 600 mg/ml L-glutamine and 50 μg/ml gentamicin. To obtain pseudoviruses, HEK293T cells were transfected simultaneously with two plasmids: pcDNA3.1env (SF162.LS) (NIH) and backbone pSG3Δenv (with the defective *env* gene) using MATra (PromoKine) according to the protocol recommended by the manufacturer. The plates were put into the CO_2-incubator and incubated at +37 °C. In 4–6 hours the medium in the plates was replaced. The cultural medium was collected after 48 hours of incubation. Pseudoviruses were separated from cells by low-frequency centrifugation followed by filtration through a nitrocellulose filter with 0.45 μm pore diameter. The obtained pseudoviral samples were stored at –80 °C in the DMEM medium containing 20 % fetal bovine serum.

Determining functional activity. Functional activity of the obtained pseudoviruses was determined using TZM-bl cells. 5×10^4 TZM-bl cells were put in each hole of a 96-hole cultural plate, then 50 μl of suspension of the analyzed env-pseudoviruses were added to each hole, four hopes per pseudovirus. The plates were incubated at 37 °C in 5 % CO_2. After 48 hours of incubation, luciferase activity was determined in cells with a LAR (Promega) set in accord with the manufacturer's recommendations. Pseudovirus-free TZM-bl cells were used as the control. A luminescent signal was measured on a STATFAX® 4400 (Lumate) luminometer and registered in RLU (relative light units), the intensity of luminescence correlates with the amount of a pseudovirus in a cell (Ryzhikov at al., 2012). Env-pseudovirus was used in further work if RLU exceeded the twofold value of spontaneous luminescence of TZM-bl cells by 50 times or more.

Electron microscopy. Cell-purified supernatant, containing pseudo-viral particles, was fixed by adding the equal volume of 8 % paraformaldehyde solution. The fixation time was 48 hours at +4 °C.

A standard negative staining was used for control counting, studying the forms and dimensions of virus-like particles (Harris, Horne, 1994). The supernatant was put on copper grids for electron microscopy, covered with carbon-stabilized formvar film. The samples were stained with 2 % aqueous solution of uranyl acetate.

The cell culture was separated from the surface with a rubber spatula and fixed in 4 % paraformaldehyde solution at +4 °C for 24 hours. Then it was rinsed in a buffer, addi-

tionally fixed with 1 % solution of osmic acid, dehydrated according to the standard procedure in increasing-concentration solutions of ethanol and acetone, and embedded in epon-araldite mixture. The procedure was described earlier in the literature (Sergeev at al., 2016). The semithin (1 μm) and ultrathin (50–60 nm) sections were prepared on a Reichert-Jung microtome (Austria). Semithin sections were stained with azure-II solution and studied in an AxioImagerZ1 light microscope (Zeiss, Germany). The ultrathin sections were stained with uranyl acetate and lead citrate and studied in a JEM 1400 electron microscope (Jeol, Japan) at accelerated voltage 80 kV. Image acquisition, image analysis and processing were performed using a Veleta digital camera (SIS, Germany) and iTEM software suit (SIS, Germany).

Pseudoviruses concentration. 20 ml of cell-purified supernatant were successively run through a Vivaspin 6 (300 000 MWCO) centrifugal concentrator (Sartorius, UK) in 5 ml doses (5 min per dose at the speed of 3000 rotations/min). We used the concentrator with a membrane filter with a 300 000 kDa molecular weight cutoff threshold, holding objects with a molecular weight higher than 300 000 kDa and letting pass macromolecules with a smaller molecular weight. Then the filter was taken out and some pieces, sized approximately 1 × 1 mm, were cut off from its different parts. Further sample preparation followed the technique described above for the cell culture. Embedding into resin, membrane pieces were oriented in such a way as to obtain a section through the entire thickness of the filter.

To determine a physical titre of pseudoviral particles in suspension, the average amount of such particles per length unit of the upper (from the side of the filtrated fluid) edge of the filter on the cutoff was counted. Based on the assumption about homogeneity of the fluid flow in the course of filtering on the filter area and the isotropy of the filter itself, it was believed that the density of particles on the filter is uniform in all directions, and, therefore, is the square of the linear density. The value was calculated for the full filter area – 4 cm^2.

Results

To count the amount of viral particles several methods can be used, including plaque-forming cells; quantitative RT-PCR; immunofluorescence microscopy; analytical flow cytometry; electron microscopy, etc. (Ferris et al., 2002; Reid et al., 2003; Malenovska, 2013; Heider, Metzner, 2014; Rossi et al., 2015).

Electron microscopy has some advantages over the above-listed methods since it gives information not only about the amount of all viral particles (regardless of whether they are infectious) but also about the morphology of an analyzed virus (Malenovska, 2013). Briefly, the method can be described as follows. An analyzed sample containing viral material is added a certain amount of suspension with a known concentration of latex beads. The mixture is put on copper grids covered with supporting film or adsorption for a particular period of time, or spraying of the mixture over the grids with an ultrasound probe. Then the electron-microscopic grids are analyzed in an electron microscope. Latex particles and viral particles are counted simultaneously in several grid elements. Knowing the latex concentration and comparing it with the amount of the detected viral particles, it is possible to estimate concentration in the initial sample. Naturally, this method is not very accurate, but convenient, and it is widely used for quantitative assessment of some viruses.

With such counting, errors are due to heterogeneous properties of the surface of the supporting film, aggregations of both latex and viral material, deviations from the experimental design, such as insufficient mixing or contamination during the spraying. The main limitation of negative staining method for counting the amount of viral particles by means of electron microscopy is insufficient sensitivity: the level of concentration required to obtain reliable results should be at least 10^7 particles/ml (Reid et al., 2003).

The study presents an original method (on the case of env-pseudoviruses) enabling to evaluate the amount of VLP in the samples, where their concentration is lower than 10^7 particles/ml.

HIV-1 virus-like particles (env-pseudoviruses) were chosen as a model. Env-pseudoviruses can penetrate into the cell similarly to live human immunodeficiency virus. Due to the deficient genome, though, they cannot form adequate virus progeny so they are safe to work with. Two types of plasmids are used to obtain env-pseudoviruses: envelope and packaging. Packaging, or core plasmid (pSG3Δenv) codes all HIV-1 proteins except the envelope ones. The second plasmid (pEnv), on the contrary, codes only HIV-1 envelope proteins. To verify infectivity of env-pseudoviruses, TZM-bl genetically engineered cells are used; their genome contains a luciferase gene that becomes active when getting to an HIV-1 or pseudovirus cell (Montefiori, 2009).

Co-transfection of 293T cells with two plasmids forms pseudoviral progeny capable of only single cells infecting, without further replication. Electronic microscopy of ultrathin sections was used to confirm pseudoviral particles assembly and yield (Fig. 1).

Pseudovirus was also characterized by defining functional activity with a single infection cycle in TZM-bl cell-target culture. The results are shown on Fig. 2. Signal intensity is given in luminescence standard units – RLU/ml. The findings are indicative of the functional activity of the virus.

However, using negative staining we did not reveal virus-like particles (VLP) with positive functional activity in the supernatant fluid. The most probable reason was a low particle concentration in the original fluid. To solve the task, we proposed a method of concentrating viral particles on polyethersulfone membranes used in centrifugal concentrators. Viral particles are deposited from suspension

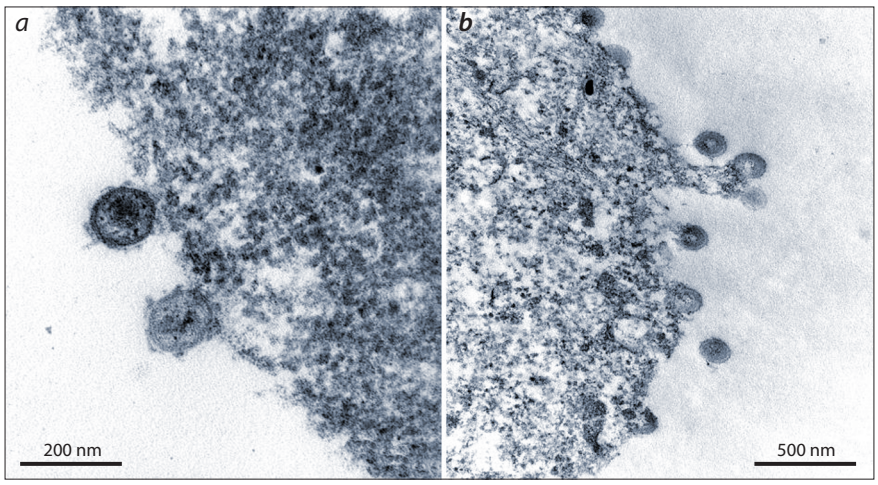

Fig. 1. *a*, Electron microscopic image of 293T cells with SF162 budded pseudovirus. *b*, Several pseudovirus particles of different maturity level.

Transmission electron microscopy, an ultrathin section.

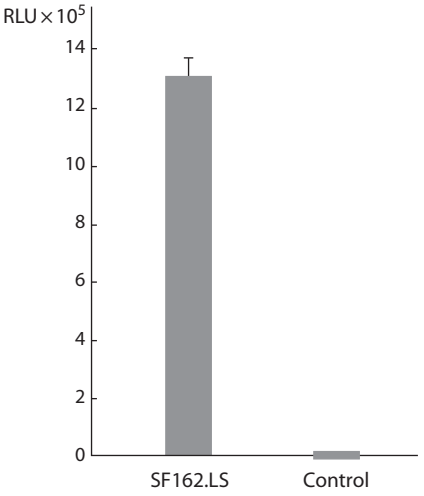

Fig. 2. Functional activity of SF162.LS pseudoviruses.

on a concentrator membrane by low-speed centrifugation. Henceforth, a part of the membrane is embedded into epon-araldite and used to make ultrathin sections for analysis with electron microscope. We used membranes with the 300 000 kDA cutoff threshold to entrap pseudoviral particles of around 100 nm diameter.

Figure 3 demonstrates VLP captured in a nanofilter during centrifugation. The particles are clearly discernable in the body and on the surface of the nanofilter, which allows to identify them morphologically and count (determine the average number per length unit of a filter section).

Assuming homogeneity of the fluid flow in the course of filtering on the filter area and the isotropy of the filter itself, we believed that the density of particles on the filter is uniform in all directions, and, therefore, is the square of the linear density. Particles were counted for the full filter area – 4 cm^2. On average, 3 virions per run-

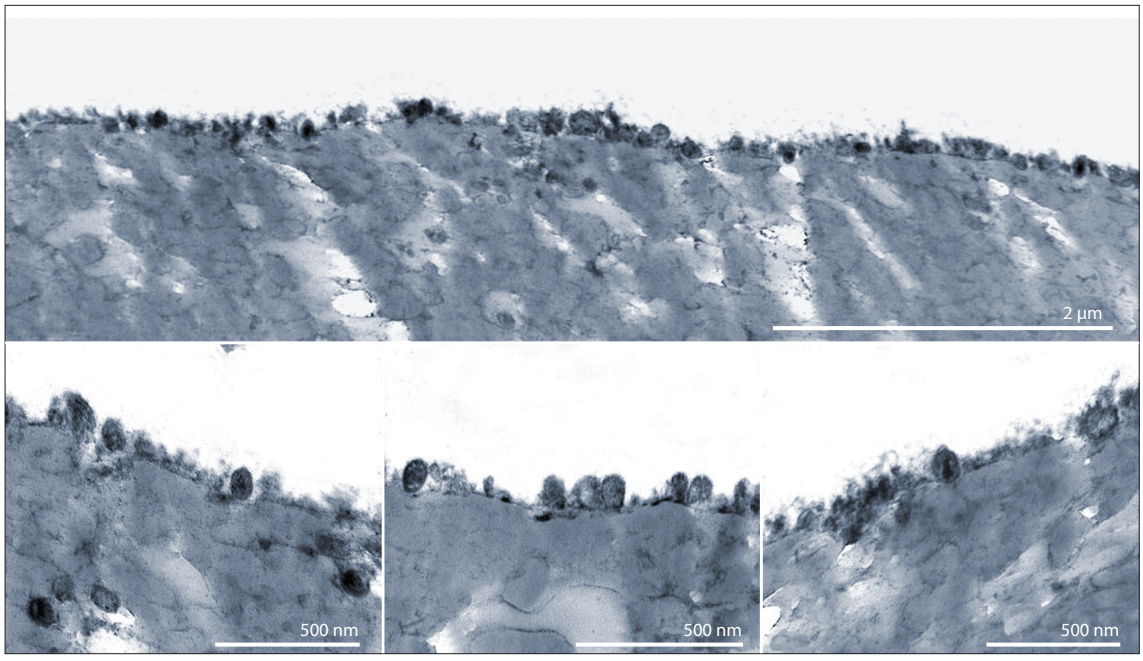

Fig. 3. Pseudovirus particles captured by a Vivaspin 6 filter (300 000 MWCO).

Transmission electron microscopy, ultrathin sections.

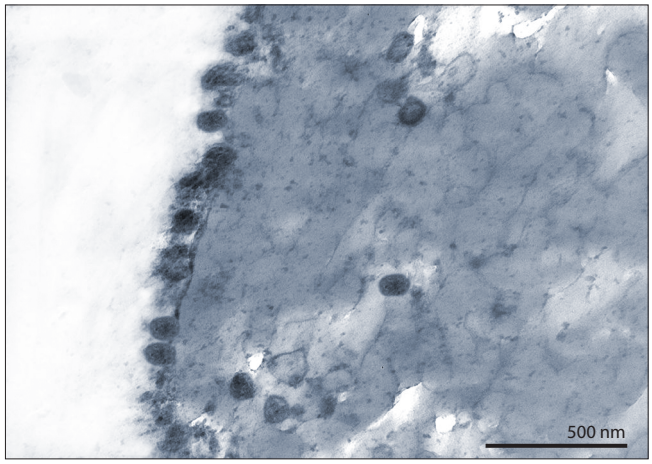

Fig. 4. Pseudovirus particles captured by a Vivaspin 6 (300 000 MWCO) filter. Particles are located both on the surface and inside the filter.
Transmission electron microscopy, ultrathin section.

ning 10 μm are observed on a section. Considering that the perpendicular density is the same, there are $3 \times 3 = 9$ virions per 100 μm^2. Thus, there are around 4×10^7 virions on the entire filter surface (the area of 4 cm^2). Understanding that the volume of supernatant containing pseudoviruses was 20 ml, the input concentration of pseudoviruses in a sample (4×10^7 particles/20 ml = 2×10^6 particles per 1 ml) can be determined. This value is by order lower than the value that can be registered when negative staining is used, which confirms our assumption (negative staining did not detect pseudoviral particles).

Conclusion

Thus, the proposed method overcomes the main disadvantage of virus quantitative estimation by means of electron microscopy, associated with a relatively high detection threshold (particles concentration – higher than 10^7/ml). As demonstrated on Fig. 4, the density of pseudoviruses on the section is quite high. It permits to expect that decreasing virion concentration in suspension by two more orders will also allow identifying the captured virions. In addition, centrifugal concentrator successively runs considerable suspension volumes through the same filter, which can also increase sensitivity of the method.

Comparing the proposed method with a widely used plaque-technique, we can emphasize the advantages related to the speed of work execution and possibility to determine the total titer of viral particles, including "noninfectious" particles. The plaque-technique detects only the particles that possess infectivity under the given conditions. In some cases, for instance, when characterizing vaccine specimens, it is necessary to know the amount of all particles, including noninfectious ones because they can also influence the host immune response (Blancett et al., 2017).

The developed method can be used for quantitative analysis of various samples containing viruses of animals, plants and humans, as well as noninfectious nanoparticles and virus-like particles. The method is especially valuable for analyzing specimens with a low content of viral particles. This approach increases sensitivity, accuracy and reproducibility of quantitative analysis made by means of electron spectroscopy.

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