Journal of Visualized Experiments

Single cell micro aspiration as an alternative strategy to fluorescence-activated cell sorting for giant virus mixture separation

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60148R2
Full Title:	Single cell micro aspiration as an alternative strategy to fluorescence-activated cell sorting for giant virus mixture separation
Keywords:	Single cell micro-aspiration; cloning; Giant viruses; Faustovirus; co-culture; Vermamoeba vermiform; Flow Cytometry; FACS
Corresponding Author:	Julien Andreani IHU Mediterranee Infection Marseille, France FRANCE
Corresponding Author's Institution:	IHU Mediterranee Infection
Corresponding Author E-Mail:	miaguiabidou@gmail.com
Order of Authors:	Dehia Sahmi-Bounsiar
	Paulo Victor de Miranda Boratto
	Graziele Pereira Oliveira
	Jacques Yaacoub Bou Khalil
	Bernard La Scola
	Julien Andreani
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Marseille, PACA, France

ΜΕΦΙ / MEPHI
Microbes, Evolution, Phylogeny and Infection

Marseille, 8th April 2019

Dear editors,

Please find our manuscript entitled "**Single cell micro aspiration as an alternative strategy to fluorescence-activated cell sorting for giant viruses mixture separation**".

In this manuscript we report the developing of a novel alternative strategy in order to separate giant viruses from our co-culture supernatants. Indeed, we used microaspiration to capture amoebas independently infected by two novel giant viruses previously unknown. We succeeded in separating 2 novel giant viruses that we named Clandestinovirus and Usurpativirus. This method appears to be an appropriate candidate to solving the non-separation and intermingling of viruses possessing relative same capsid size or relative similar genomic contents. In addition, they could help teams in the future to resolve similar culture problems.

We sincerely hope that this **method** is relevant and could be published in Journal Of Visual Experiments.

Best regards

Julien Andreani and Bernard La Scola

MEPHI, IHU Fondation Méditerranée Infection, 19-21 boulevard Jean Moulin, 13005 Marseille, France
Tel: +33 (0)4 13 73 24 01 - Fax: +33 (0)4 13 73 24 02 – bernard.la-scola@univ-amu.fr

TITLE:

Single Cell Micro-Aspiration as an Alternative Strategy to Fluorescence-Activated Cell Sorting for Giant Virus Mixture Separation

AUTHORS AND AFFILIATIONS:

Dehia Sahmi-Bounsiar[1], Paulo Victor de Miranda Boratto[1], Graziele Pereira Oliveira[1], Jacques Yaacoub Bou Khalil[2], Bernard La Scola[1,2], Julien Andreani[1]

[1]Microbes, Evolution, Phylogeny and Infection (MEΦI), Aix-Marseille Université UM63, Institut de Recherche pour le Développement IRD 198, Assistance Publique – Hôpitaux de Marseille (AP-HM), Marseille, France.

[2]Institut Hospitalo-Universitaire (IHU) - Méditerranée Infection, Marseille, France

Corresponding Authors:

Bernard La Scola (Bernard.la-scola@univ-amu.fr)

Julien Andréani (miaguiabidou@gmail.com)

Email Addresses of Co-authors:

Dehia Sahmi-Bounsiar (medecine.to@hotmail.com)

Boratto Paulo (pvboratto@gmail.com)

Oliveira Graziele (graziufmg2008@gmail.com)

Jacques Abou Khalil (boukhaliljacques@gmail.com)

KEYWORDS:

single cell micro-aspiration, cloning, giant viruses, Faustovirus, co-culture, *Vermamoeba vermiformis*, flow cytometry, FACS.

SUMMARY:

Here, we describe a single cell micro-aspiration method for the separation of infected amoebae. In order to separate viral subpopulations in *Vermamoeba vermiformis* infected by Faustoviruses and unknown giant viruses, we developed the protocol detailed below and demonstrated its ability to separate two low-abundance novel giant viruses.

ABSTRACT:

During the amoeba co-culture process, more than one virus may be isolated in a single well. We previously solved this issue by end point dilution and/or fluorescence activated cell sorting (FACS) applied to the viral population. However, when the viruses in the mixture have similar morphologic properties and one of the viruses multiplies slowly, the presence of two viruses is discovered at the stage of genome assembly and the viruses cannot be separated for further characterization. To solve this problem, we developed a single cell micro-aspiration procedure that allows for separation and cloning of highly similar viruses. In the present work, we present how this alternative strategy allowed us to separate the small viral subpopulations of Clandestinovirus ST1 and Usurpativirus LCD7, giant viruses that grow slowly and do not lead to amoebal lysis compared to the lytic and fast-growing Faustovirus. Purity control was assessed by

specific gene amplification and viruses were produced for further characterization.

INTRODUCTION:

Nucleocytoplasmic large DNA viruses (NCLDV) are extremely diverse, defined by four families that infect eukaryotes[1]. The first described viruses with genomes above 300 kbp were *Phydcodnaviridae*, including *Paramecium bursaria Chlorella virus 1* PBCV1[2]. The isolation and the first description of Mimivirus, showed that the size of viruses doubled in terms of both the size of the particle (450 nm) and the length of the genome (1.2 Mb)[3]. Since then, many giant viruses have been described, usually isolated using an amoeba co-culture procedure. Several giant viruses with different morphologies and genetic contents can be isolated from *Acanthamoeba* sp. cells, including Marseilleviruses, Pandoraviruses, Pithoviruses, Mollivirus, Cedratviruses, Pacmanvirus, Tupanvirus, and recently Medusavirus[4-17]. In parallel, the isolation of *Vermamoeba vermiformis* allowed the isolation and description of the giant viruses Faustovirus, Kaumoebavirus, and Orpheovirus[18-20]. Other giant viruses were isolated with their host protists, such as *Cafeteria roenbergensis*[21], *Aureococcus anophagefferens*[22], *Chrysochromulina ericina*[23], and *Bodo saltans*[24]. All of these isolations were the result of an increasing number of teams working on isolation and the introduction of high throughput strategy updates[25-28], such as the improvement of the co-culture system with the use of flow cytometry.

In 2016, we used a strategy associating co-culture and flow cytometry to isolate giant viruses[27]. This strategy was developed to increase the number of samples inoculated, to diversify protists used as cell supports, and to quickly detect the lysis of the cell support. The system was updated by adding a supplemental step to avoid preliminary molecular biology identification and quick detection of an unknown viral population as in the case of Pacmanvirus[29]. Coupling flow cytometry to cell sorting allowed for separation of a mixture of Mimivirus and Cedratvirus A11[30]. However, we later encountered the limitations of the separation and detection of these viral subpopulations by flow cytometry. After sequencing, when we assembled the genomes of Faustovirus ST1[25] and Faustovirus LCD7 (unpublished data), we surprisingly found in each assembly two supplemental genomes of two novel viruses not identified in public genome databases. However, neither flow cytometry nor transmission electronic microscopy (TEM) showed that the amoebaes were infected by two different viruses, Clandestinovirus ST1 and Usurpativirus LCD7. We designed specific PCR systems to amplify Faustovirus, Usurpativirus, and Clandestinovirus markers respectively based on their genomes; our purpose was to have PCR-based systems that enable verification of the purity of the viruses being separated. However, end-point dilution and flow cytometry failed to separate them. The isolation of this single viral population was difficult because neither the morphology nor replicative elements of Clandestinovirus and Usurpativirus populations have been characterized. We detected only one viral population by flow cytometry due to the overlapping of the two populations (tested after the effective separation). We tried to separate them using single particle sorting on 96-well plates, but we did not observe any cytopathic effects, and we detected neither Clandestinovirus nor Usurpativirus by PCR amplification. Finally, it was only the combination of end point dilution followed by single amoeba micro-aspiration that enabled separation of these two low-abundance giant viruses from Faustoviruses. This method of separation is the object of this article.

PROTOCOL:

1. Amoeba culture

1.1. Use *Vermamoeba vermiformis* (strain CDC19) as a cell support.

1.2. Add 30 mL of protease-peptone-yeast extract-glucose medium (PYG) (**Table 1**) and 3 mL of the amoebae at a concentration of 1 x 10^6 cells/mL in a 75 cm^2 cell culture flask.

1.3. Maintain the culture at 28 °C.

1.4. After 48 h, quantify the amoebae using counting slides.

1.5. To rinse, harvest the cells at a concentration of 1 x 10^6 cells/mL and pellet the amoebae by centrifugation at 720 x *g* for 10 min. Remove the supernatant and resuspend the pellet in the appropriate volume of starvation medium to obtain 1 x 10^6 cells/mL (**Table 1**).

2. Propagation of stock virus in amoebae

NOTE: Before dilution, it is important to culture the stock sample to obtain enough fresh culture, then proceed to filtration.

2.1. Use 1 x 10^6 of amoeba culture in starvation medium.

2.2. Inoculate the mixture of viruses issued from the stock solution (Faustovirus/Usurpativirus LCD7 or Faustovirus/Clandestinovirus ST1) after the co-culture process on the cell support at a multiplicity of infection (MOI) of 0.01.

NOTE: The MOI is important to reduce the abundance of the major viral population and the number of infected cells.

2.3. Incubate at 30 °C until cytopathic effects (CPE) are induced, such as amoebal rounding or lysis, approximately 10 to 14 h after infection.

2.4. Collect the media and filtrate through a 5 µm filter to remove cellular debris.

3. End-point dilution

3.1. Perform a serial dilution (10^{-1} to 10^{-11}) of the viral sample in starvation medium (**Table 1**).

3.2. Inoculate 2 mL of 1 x 10^6 *Vermamoeba vermiformis* contained in each Petri dish with 100 µL of the mixture inoculum.

3.3. Place the Petri dishes into a sealable plastic bag at 30 °C.

3.4. Begin observing the Petri dishes with inverted optical microscopy at 6 h postinfection and check cell morphology every 4 to 8 h.

3.5. At the appearance of the cytopathic effect characterized by rounding cells, begin the single cell micro-aspiration process.

4. Single cell micro-aspiration

4.1. Prepare the host.

NOTE: This preparation is made for the release of infected single cells to a fresh cell support.

4.1.1. Treat the amoebae in the culture with an antimicrobial agent containing 10 µg/mL of vancomycin, 10 µg/mL of imipenem, 20 µg/mL of ciprofloxacin, 20 µg/mL of doxycycline, and 20 µg/mL of voriconazole. This mixture is used to avoid bacterial and fungal contamination.

NOTE: The procedure takes place on a bench outside the microbiological safety station. Add 2 mL of amoebae concentrated at 1 x 10^6 cells/mL each into 15 Petri dishes. For amoeba adherence, incubate the culture at 30 °C for 30 min.

4.2. Select the Petri dish used for the micro-aspiration from the limit dilution according to the following criteria: 1) absence of any visible contamination by fungal and bacterial agents, 2) evidence of cytopathic effect of amoebae due to the viruses, and 3) prelysis and rounding phase of the amoebae (to avoid aspiration of viral particles).

4.3. Set up a workstation with the following materials (see **Figure 1A,B**):
Micromanipulator, which allows microcapillary positioning;
Manual control pressure device, used to aspirate and release the cells into the microcapillary;
Inverted microscope;
Plug and play motor modules;
Camera;
Computer module to visualize manipulation and take pictures.

4.4. Choose a microcapillary (see **Figure 1C**).

NOTE: The size of the cells, the deformation and adhesion of their membranes to the surfaces, and the cellular motility can impact the smooth progress of the micro-aspiration. The microcapillary diameter can be precisely chosen and adapted to specific cell types depending on their sizes and methods of aspiration. A microcapillary of 20 µm inner diameter was used to aspirate a rounding amoeba (diameter ~10 µm). This allows the upkeep of an internal position and an easy release of the cell.

4.5. Mount the system.

4.5.1. Fix the operating angle of the gripping system on the motorized module at 45°.

4.5.2. Perform a double installation, first on the gripping system, and then on the microcapillary.

4.5.3. Focus on the cells after running a few drops of oil through the microcapillary.

NOTE: The mineral oil with biological compatibility is supplied by the device.

4.5.4. Complete mounting following manufacturer's recommendations.

4.6. Clone cells (see **Figure 2A,B**).

NOTE: This procedure is similar to the one described by Fröhlich and König[31].

4.6.1. Place the Petri dish containing 2 mL of infected amoebae under the microscope.

4.6.2. Focus first on the cells, and then on the microcapillary immersed in the culture.

4.6.3. Pick a rounded single cell and bring the microcapillary closer to the micromanipulator.

4.6.4. Exert soft aspiration with manual pressure control on the cell, taking it inside the microcapillary. Remove the single cell from the first sample and release in the cellular support, then incubate it at 30 °C.

4.6.5. Conduct daily observations with an inverted optical microscope to observe the appearance of the cells and to monitor the emergence of the cytopathic effect.

5. PCR screening

NOTE: Following step 4, a systematic screening by PCR is crucial to confirm the separation. In both Usurpativirus/Faustovirus and Clandestinovirus/Faustovirus, the design and application of the specific primer and probe systems were done using Primer-BLAST online[32] (**Table 2**).

5.1. Extract DNA from a part of the positive culture samples (i.e., where a cytopathic effect is observed), using an automated extraction system according to the manufacturer's protocol.

5.2. Use appropriately designed primers.

NOTE: Here we designed primers to amplify core genes annotated as RpB2 (Faustovirus), LCD7 major capsid protein (Usurpatvirus) and minor capsid protein (Clandestinovirus)

5.3. Perform standard PCR using a thermocycler.

5.3.1. Carry out 20 µL PCR reactions with 50 µM of each primer (**Table 2**), 1x Master Mix, and

RNase free water.

5.3.2. Activate the Taq DNA polymerase for 5 min at 95 °C, then follow with 45 cycles of 10 s denaturation at 95 °C, annealing of the primers for 30 s at 58 °C, and extension for 30 s at 72 °C.

5.4. Run the PCR products on a 1.5% agarose gel, stain with DNA gel stain (**Table of Materials**), and visualize with UV.

6. Virus production and purification

6.1. Put the rest of the Petri dish culture back in a small flask.

6.2. For the virus production, prepare 15 flasks of 145 cm^2, containing 40 mL of *Vermamoeba vermiformis* in starvation medium and 5 mL of the isolated virus already transferred from the Petri dish to small flasks.

6.3. Treat with the same antibiotic and antifungal mixture used in step 4.1.

6.4. Incubate at 30 °C. Observe every day with inverted optical microscopy.

6.5. After the complete infection, pool all flasks. Use a 0.45 µm filter to eliminate debris.

6.6. Ultracentrifuge all supernatants at 50,000 x *g* for 45 min.

6.7. After centrifugation, remove the supernatant from each tube by aspiration and resuspend the pellet in 1 mL of phosphate buffered saline (PBS).

6.8. Purify the virus produced using 25% sucrose (27.5 g sucrose in 100 mL of PBS, sterilized by filtration).

6.9. Centrifuge 8 mL of sucrose and 2 mL of the viral suspension at 80,000 x *g* for 30 min. Resuspend the viral pellet in 1 mL of PBS. Store it at -80 °C.

7. Negative staining and transmission electron microscopy

NOTE: Bou Khalil et al. previously published this protocol[27].

7.1. Deposit 5 µL of the lysis supernatant onto the glow-discharged grid. Leave for approximately 20 min at room temperature.

NOTE: The glow-discharge allows us to obtain a hydrophilic grid by plasma application.

7.2. Dry the grid carefully and deposit a small drop of 1% ammonium molybdate on it for 10 s. Leave the grid to dry for 5 min.

7.3. Proceed to electron microscopy observations at 200 keV.

8. Characterization of Clandestinovirus ST1 and Usurpativirus LCD7

8.1. Characterize pure populations of Clandestinovirus ST1 and Usurpativirus LCD7 using genome sequencing, genome assembly, bioinformatics analyses, and study of their replicative cycle as we have done for other viruses[10,20,29].

REPRESENTATIVE RESULTS:

Single cell micro-aspiration is a micromanipulation process optimized in this manuscript (**Figure 1**). This technique enables capture of a rounded, infected amoeba (**Figure 2A**) and its release in a novel plate containing uninfected amoebae (**Figure 2**). It is a functional prototype that applies to the co-culture system and has successfully isolated non-lytic giant viruses. This approach was used for the first time in the field of giant viruses and made it possible to isolate two new low-abundance giant viruses. We named the new viruses Clandestinovirus ST1 and Usurpativirus LCD7, that were in low abundance compared to the high abundance Faustoviruses. In order to analyze the viral presence in each plate after the micro-aspiration procedure, we applied PCR on the 15 micro-aspirations. We observed pure Usurpativirus (presence of Usurpativirus LCD7 [+] and absence of Faustovirus [–]) only in clone 7 (**Figure 3**). The purity of the clone was confirmed by PCR with specific protocols targeting Clandestinovirus ST1 and Usurpativirus LCD7 (**Table 2**). Electron microscopy revealed the appearance of Clandestinovirus ST1 and Usurpativirus LCD7 (**Figure 4**), which have a typical icosahedral morphology without fibrils and an icosahedral capsid of about 250 nm, respectively. After the confirmation of the production purity, the clonal virus was produced and purified for whole genome sequencing and further characterization, especially transmission electronic microscopy (TEM) for multiplication cycle studies. We confirmed the overlapping of populations (Faustovirus/novel virus) by flow cytometry (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Materials for micromanipulation. (**A**) Actual setup of the workstation. (**B**) Schematic illustration of the workstation's components. (**C**) Schematic illustration of the microcapillary.

Figure 2: Micro-aspiration steps. (**A**) Single cell isolation procedure (zoom x 40). (**B**) Schematic illustration of the different steps of single cell aspiration: 1) Localization of the cell. 2) Aspiration of the cell. 3) Release step. The black arrows show the microcapillary and the white ones show the single cell.

Figure 3: PCR screening and confirmation. Screening PCR of micro-aspirations carried out for Usurpativirus LCD7 and Faustovirus. Presence of Usurpativirus LCD7 DNA (+) and absence of Faustovirus DNA (–) observed for clone 7 after micro-aspiration procedure.

Figure 4: **Negative staining micrograph.** Negative staining of the viral suspension, showing pure Clandestinovirus ST1 (**A**) and Usurpativirus LCD7 (**B**).

Figure 5: Representative gate plots. (**A**) Superimposition of each single viral population previously stained with fluorescent molecular probes for virus DNA labelling (following previously described protocol[26,30]). The left part of the picture shows the superposition of five strains of Faustovirus (dark green, light green, orange, red, and blue). On the right, the superposition of Faustovirus ST1 and Clandestinovirus ST1 (light purple and dark purple) is visible. (**B**) Left: The presence of two viral population of Faustovirus and Usurpativirus in the same gate as Faustovirus. Right: A dot plot of pure Usurpativirus shows the same gate defined previously for Faustovirus.

DISCUSSION:

The duration of the single cell micro-aspiration handling and its good functioning is operator-dependent. The different steps of the experiment require precision. The use of the micromanipulation components of the workstation must be under constant control by observing the process of micro-aspiration and the release of the cell. The follow-up by microscopic observation is necessary for capture and transfer of a cell. An experienced operator can take 1 to 2 h to isolate 10 cells and retransfer them one by one depending on the abundance of viruses to be isolated. The number of manipulations can vary. We advise beginning with 10 manipulations. By definition, we do not know the intrinsic characteristics of the unknown virus. Thus, the use and development of different strategies for isolation are necessary to optimize the success of separating these viruses.

The micro-aspiration process is performed under unsterile conditions (on a bench outside the microbiological safety station) and thus restricts its usage and prevents its application for the study of human pathogens. Therefore, the use of a mixture of antibiotics and antifungals to limit contaminants is mandatory. Another limitation of the method is that it can only be performed on microorganisms observable by light microscopy on which the micromanipulation components were mounted, thus, conceptually eliminating any work on microorganisms not observable by light microscopy. However, we were able to separate giant viruses of about 200 nm which remain invisible under the light microscope by using an indirect strategy consisting of separating and cloning the infected hosts.

The development of single cell micro-aspiration for amoebal capture is a part of the development of population-sorting methods. Single cell micro-aspiration allowed us to isolate two new giant viruses, Clandestinovirus ST1 and Usurpativirus LCD7, with characteristics distinctive from Faustoviruses. The highly similar morphological properties of both Clandestinovirus ST1 and Usurpativirus LCD7 to Faustovirus LCD7 and their difference of replication shows the limit of FACS, which is usually used in giant viruses sorting. It is represented by the superposition of two viral populations, Clandestinovirus ST1 and Faustovirus ST1 (**Figure 5A**) and also by the detection for Usupartivirus LCD7 and Faustovirus LCD7. However, with this new method, the entire manipulation is under visual control with careful monitoring of the cell and its integrity even after its release. The use of flow cytometry to directly sort infected amoebae could be an alternative solution to indirectly sort novel viruses. This method is usually followed by the screening of the plate in order to detect cytopathic effects or lysis. The presence of non-lytic viruses in the mix could represent a limit to amoebal sorting. However, this was not explored in the creation of this protocol and for these two novel isolations.

Beyond the application of the micromanipulation in the positioning and holding of cells in general and oocytes for intracytoplasmic sperm injection (ICSI)[33], single cell micro-aspiration has proven to be a practical method for the isolation of single prokaryotic cells observable under an optical microscope[31]. Other applications could be tested, including cell sorting on a morphological basis to have different pure samples from a mixed sample of microorganisms. The observable separation of microorganisms can be envisioned using the strategy described above.

ACKNOWLEDGMENTS:

The authors would like to thank both Jean-Pierre Baudoin and Olivier Mbarek for their advice and Claire Andréani for her help in English corrections and modifications.

This work was supported by a grant from the French State managed by the National Research Agency under the "Investissements d'avenir (Investments for the Future)" program with the reference ANR-10-IAHU-03 (Méditerranée Infection) and by Région Provence Alpes Côte d'Azur and European funding FEDER PRIMI.

DISCLOSURES:

All authors have nothing to disclose.

REFERENCES:

1. Iyer, L. M., Aravind, L., Koonin, E. V. Common Origin of Four Diverse Families of Large Eukaryotic DNA Viruses. *Journal of Virology*. **75** (23), 11720–11734 (2001).
2. Li, Y. et al. Analysis of 74 kb of DNA located at the right end of the 330-kb chlorella virus PBCV-1 genome. *Virology*. **237** (2), 360–377 (1997).
3. Scola, B. L. A Giant Virus in Amoebae. *Science*. **299** (5615), 2033 (2003).
4. Philippe, N. et al. Pandoraviruses: amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes. *Science*. **341** (6143), 281–286 (2013).
5. Antwerpen, M. H. et al. Whole-genome sequencing of a pandoravirus isolated from keratitis-inducing acanthamoeba. *Genome Announcements*. **3** (2), e00136–15 (2015).
6. Legendre, M. et al. Thirty-thousand-year-old distant relative of giant icosahedral DNA viruses with a pandoravirus morphology. *Proceedings of the National Academy of Sciences of the United States of America*. **111** (11), 4274–4279 (2014).
7. Legendre, M. et al. In-depth study of Mollivirus sibericum, a new 30,000-y-old giant virus infecting Acanthamoeba. *Proceedings of the National Academy of Sciences of the United States of America*. **112** (38), E5327–335 (2015).
8. Legendre, M. et al. Diversity and evolution of the emerging Pandoraviridae family. *Nature Communications*. **9** (1), 2285 (2018).
9. Aherfi, S. et al. A Large Open Pangenome and a Small Core Genome for Giant Pandoraviruses. *Frontiers in Microbiology*. **9**, 166 (2018).
10. Andreani, J. et al. Cedratvirus, a Double-Cork Structured Giant Virus, is a Distant Relative of Pithoviruses. *Viruses*. **8** (11), 300 (2016).
11. Rodrigues, R. A. L. et al. Morphologic and genomic analyses of new isolates reveal a second lineage of cedratviruses. *Journal of Virology*. JVI.00372–18 (2018).
12. Bertelli, C. et al. Cedratvirus lausannensis- digging into Pithoviridaediversity.

Environmental Microbiology. (2017).
13. Levasseur, A. et al. Comparison of a modern and fossil Pithovirus reveals its genetic conservation and evolution. *Genome Biology and Evolution*. evw153 (2016).
14. Dornas, F. P. et al. A Brazilian Marseillevirus Is the Founding Member of a Lineage in Family Marseilleviridae. *Viruses*. **8** (3), 76 (2016).
15. Yoshikawa, G., Blanc-Mathieu, R. et al. Medusavirus, a novel large DNA virus discovered from hot spring water. *Journal of Virology*. JVI.02130–18 (2019).
16. Legendre, M. et al. Pandoravirus Celtis Illustrates the Microevolution Processes at Work in the Giant Pandoraviridae Genomes. *Frontiers in Microbiology*. **10**, 430 (2019).
17. Abrahão, J. et al. Tailed giant Tupanvirus possesses the most complete translational apparatus of the known virosphere. *Nature Communications*. **9** (1), 749 (2018).
18. Reteno, D. G. et al. Faustovirus, an asfarvirus-related new lineage of giant viruses infecting amoebae. *Journal of Virology*. **89** (13), 6585–6594 (2015).
19. Bajrai, L. et al. Kaumoebavirus, a New Virus That Clusters with Faustoviruses and Asfarviridae. *Viruses*. **8** (12), 278 (2016).
20. Andreani, J. et al. Orpheovirus IHUMI-LCC2: A New Virus among the Giant Viruses. *Frontiers in Microbiology*. **8**, 779 (2018).
21. Fischer, M. G., Allen, M. J., Wilson, W. H., Suttle, C. A. Giant virus with a remarkable complement of genes infects marine zooplankton. *Proceedings of the National Academy of Sciences of the United States of America*. **107** (45), 19508–19513 (2010).
22. Moniruzzaman, M. et al. Genome of brown tide virus (AaV), the little giant of the Megaviridae, elucidates NCLDV genome expansion and host-virus coevolution. *Virology*. **466-467**, 60–70 (2014).
23. Gallot-Lavallée, L., Blanc, G., Claverie, J.-M. Comparative genomics of Chrysochromulina Ericina Virus (CeV) and other microalgae-infecting large DNA viruses highlight their intricate evolutionary relationship with the established Mimiviridae family. *Journal of Virology*. JVI.00230–17 (2017).
24. Deeg, C. M., Chow, C.-E. T., Suttle, C. A. The kinetoplastid-infecting Bodo saltans virus (BsV), a window into the most abundant giant viruses in the sea. *eLife*. **7**, 235 (2018).
25. Boughalmi, M. et al. High-throughput isolation of giant viruses of the Mimiviridae and Marseilleviridae families in the Tunisian environment. *Environmental Microbiology*. **15** (7), 2000–2007 (2013).
26. Khalil, J. Y. B. et al. High-Throughput Isolation of Giant Viruses in Liquid Medium Using Automated Flow Cytometry and Fluorescence Staining. *Frontiers in Microbiology*. **7** (120), 26 (2016).
27. Bou Khalil, J. Y., Andreani, J., Raoult, D., La Scola, B. A Rapid Strategy for the Isolation of New Faustoviruses from Environmental Samples Using Vermamoeba vermiformis. *Journal of Visualized Experiments : JoVE*. (112), e54104–e54104 (2016).
28. Khalil, J. Y. B., Andreani, J., La Scola, B. Updating strategies for isolating and discovering giant viruses. *Current Opinion in Microbiology*. **31**, 80–87 (2016).
29. Andreani, J. et al. Pacmanvirus, a new giant icosahedral virus at the crossroads between Asfarviridae and Faustoviruses. *Journal of Virology*. JVI.00212–17 (2017).
30. Khalil, J. Y. B. et al. Flow Cytometry Sorting to Separate Viable Giant Viruses from Amoeba Co-culture Supernatants. *Frontiers in Cellular and Infection Microbiology*. **6**, e17722

(2017).
31. Fröhlich, J., König, H. New techniques for isolation of single prokaryotic cells. *FEMS Microbiology Reviews*. **24** (5), 567–572 (2000).
32. Ye, J. et al. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. **13** (1), 134 (2012).
33. Kimura, Y., Yanagimachi, R. Intracytoplasmic sperm injection in the mouse. *Biology of Reproduction*. **52** (4), 709–720 (1995).

Figure 1

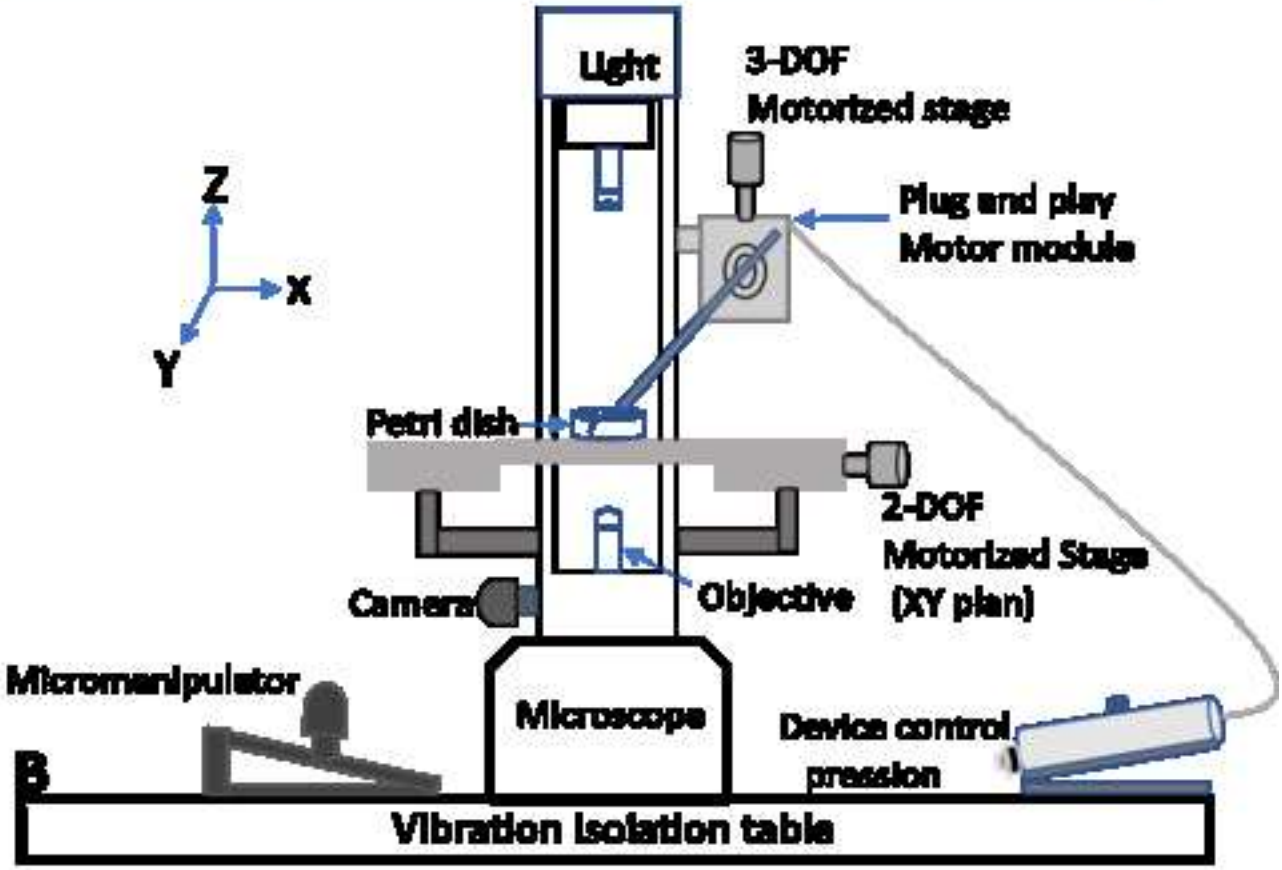
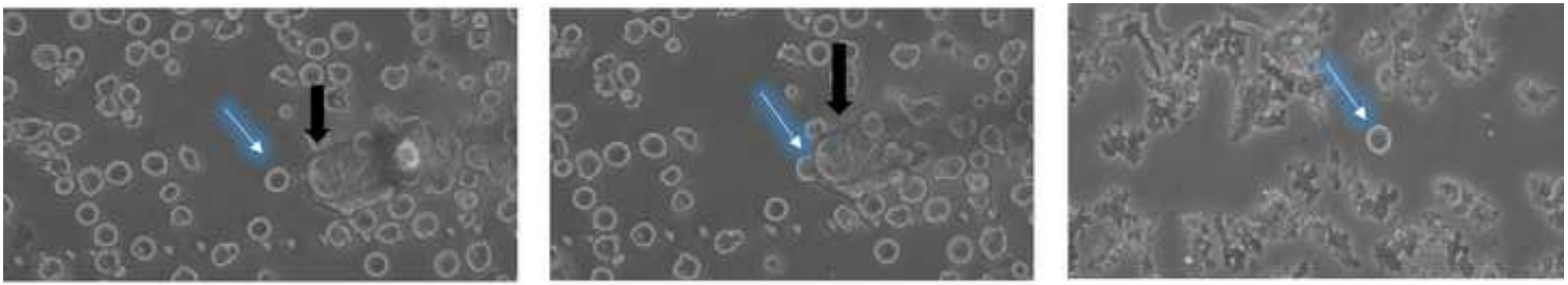
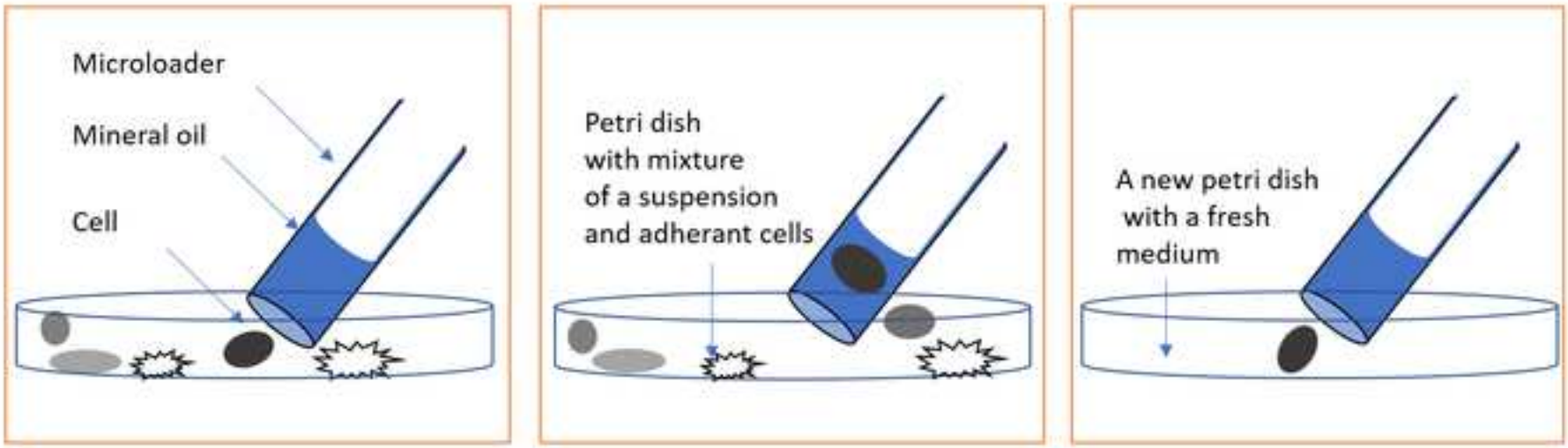


Figure 2



A (1) (2) (3)



B (1) (2) (3)

Figure 3

Click here to access/download;Figure;Image3.DPI_300.tif

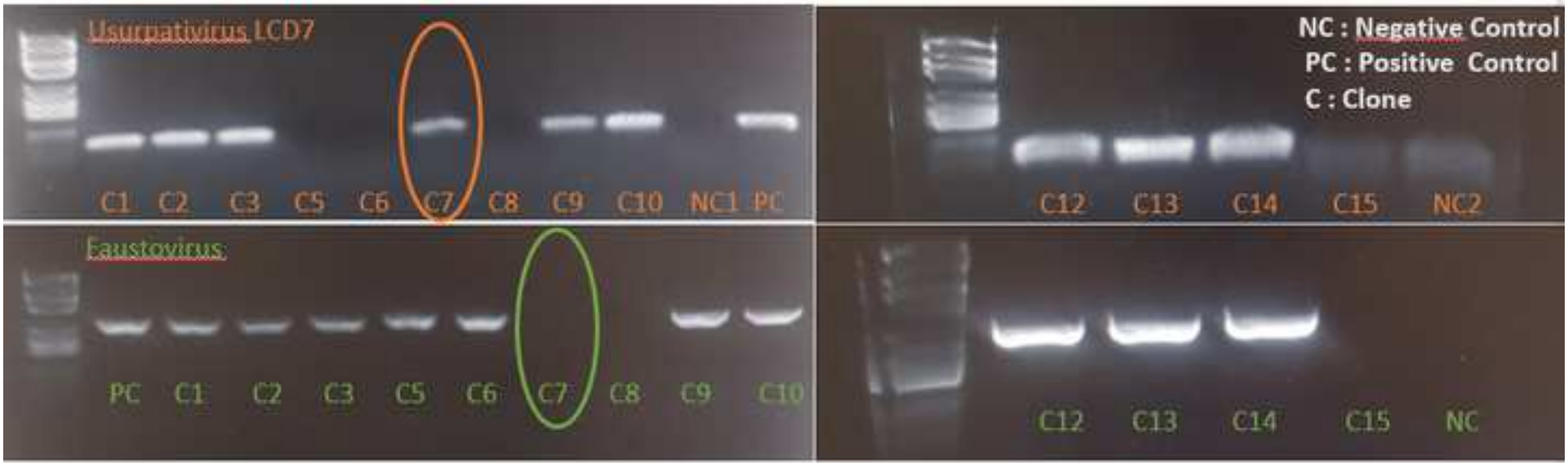


Figure 4

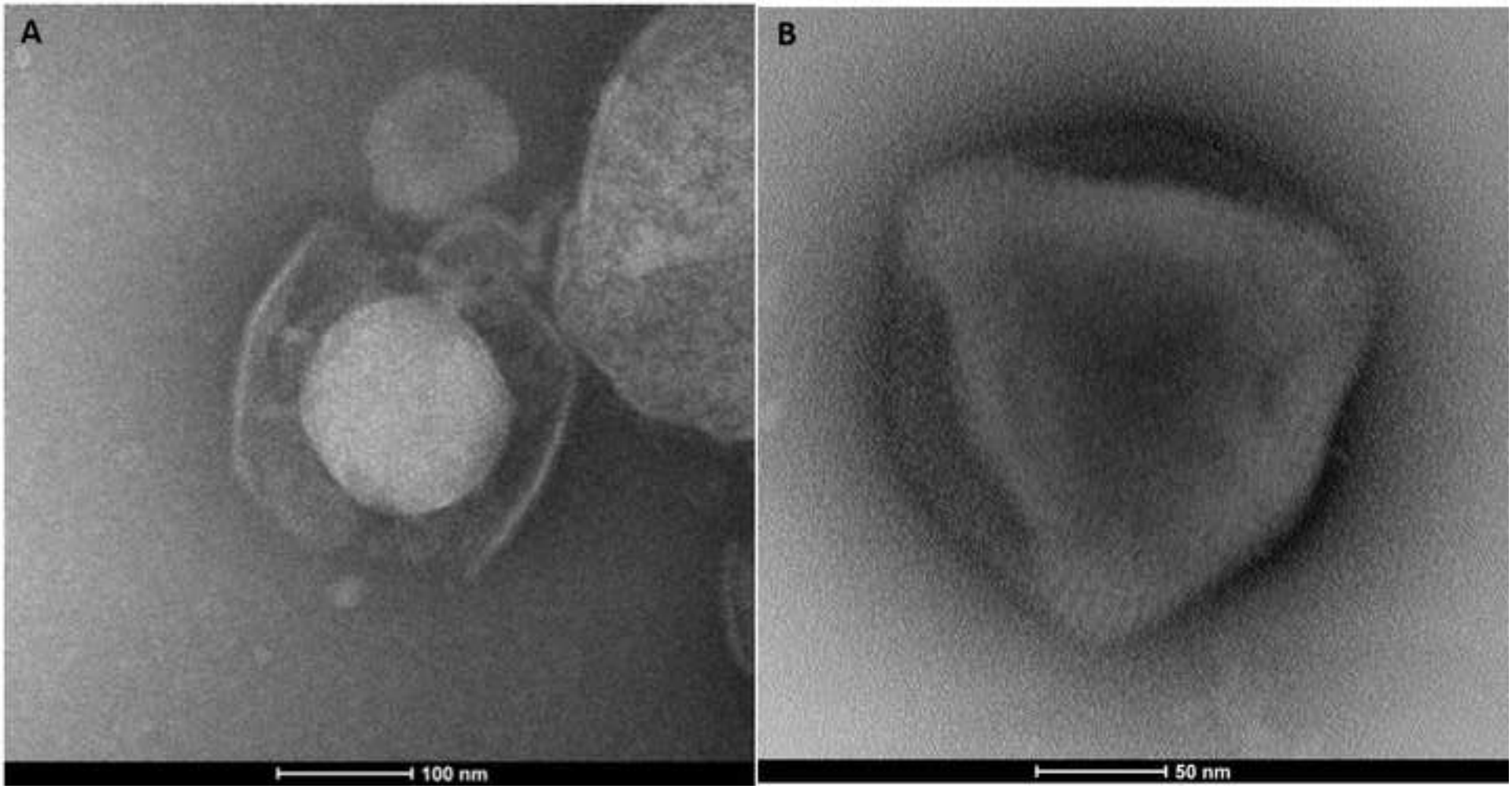
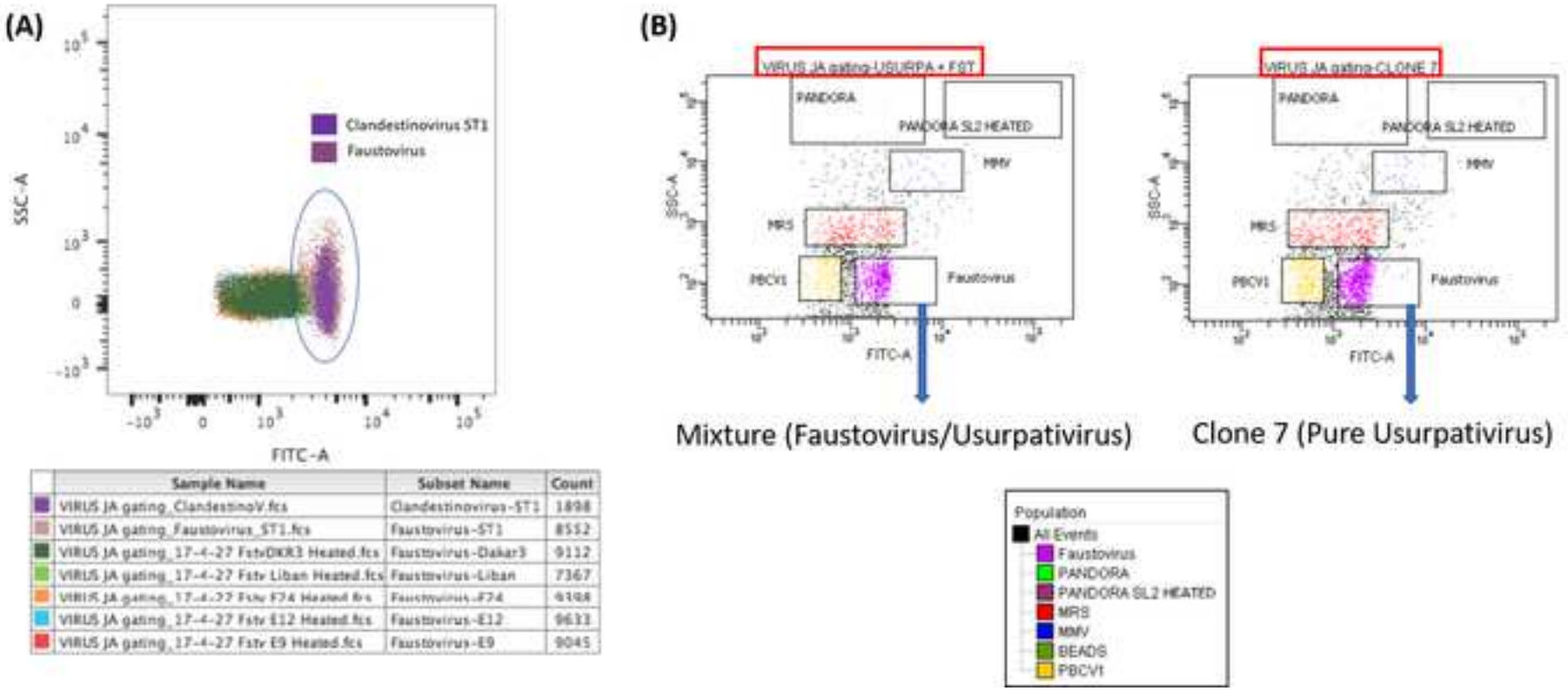


Figure 5

Click here to access/download;Figure;Figure 5 finale.tif



	Sample Name	Subset Name	Count
	VIRUS JA gating_ClandestinoV.fcs	Clandestinovirus-ST1	1898
	VIRUS JA gating_Faustovirus_ST1.fcs	Faustovirus-ST1	8552
	VIRUS JA gating_17-4-27 FstvDKR3 Heated.fcs	Faustovirus-Dakar3	9112
	VIRUS JA gating_17-4-27 Fstv Liban Heated.fcs	Faustovirus-Liban	7367
	VIRUS JA gating_17-4-27 Fstv F74 Heated.fcs	Faustovirus-F74	9198
	VIRUS JA gating_17-4-27 Fstv E12 Heated.fcs	Faustovirus-E12	9633
	VIRUS JA gating_17-4-27 Fstv E9 Heated.fcs	Faustovirus-E9	9045

Table 1

Click here to access/download;Table;Table 1. Composition of culture media.xlsx

PYG composition	**quantities**
Proteose peptone	20 g
Yeast extract	20 g
MgSO4. 7H2O	0.980 g
CaCl2	0.059 g
Citrate sodium. Dihydrate	1 g
Fe (NH4) 2(SO4) 2 x 6 H2O	0.02 g
Glucose	18 g
Distilled water	1 L
Adjust pH at 6.8 with HCl or KOH	
Autoclave 15 min at 121 °C	

Starvation medium	**quantites**
Yeast extract	2 g
Glucose	18 g
Fe (NH4) 2(SO4) 2 . 6 H2O	0.02 g
PAS (detailed below)	1 L

PAS solution A	**quantites**
KH2PO4	0.136 g
Na2HPO4	0.142 g
PAS solution B	**quantites**
MgSO4.7H2O	4.0 mg
CaCl2.2H2O	4.0 mg
NaCl	0.120 g

10 mL each of solution A and B are added into 1 L of distilled water.

Table 2

Virus target	*Target gene*
Faustovirus RpB2	**RpB2**
Usurpatvirus LCD7 Major capsid gene	**Major capsid gene**
Clandestinovirus ST1 Minor capsid gene	**Minor capsid gene**

Foward primer (5->3)
CWCAATCHGCYGATACHGGTGA
GGGCAAGAAGCTCCAAGCTA
AAAATGAACGCGTTGGAGGC

Reverse primer (5->3)
TGATTWGCYAATGGNGCYGC
GGGTTGAGGAGGAGTCAACG
ACCGGCGAATGTTCCTATGG

Table of Materials

Name of Material/Equipment	Company	Catalog Number
Agarose Standard	Euromedex	Unkown
AmpliTaq Gold 360 Master Mix	Applied Biosystems	4398876
CellTram 4r Oil	Eppendorf	5196000030
Corning cell culture flasks 150 cm2	Sigma-aldrich	CLS430825
Corning cell culture flasks 25 cm2	Sigma-aldrich	CLS430639
Corning cell culture flasks 75 cm2	Sigma-aldrich	CLS430641
DFC 425C camera	LEICA	Unkown
Eclipse TE2000-S Inverted Microscope	Nikon	Unkown
EZ1 advanced XL	Quiagen	9001874
Glasstic Slide 10 With Counting Grids	Kova International	87144E
Mastercycler nexus	Eppendorf	6331000017
Microcapillary 20 µm	Eppendorf	5175 107.004
Micromanipulator InjectMan NI2	Eppendorf	631-0210
Nuclease-Free Water	ThermoFischer	AM9920
Optima XPN Ultracentrifuge	BECKMAN COULTER	A94469
Petri dish 35 mm	Ibidi	81158
Sterile syringe filters 5 µm	Sigma-aldrich	SLSV025LS
SYBR green Type I	Invitrogen	unknown
SYBR Safe	Invitrogen	S33102
Tecnai G20	FEI	Unkown
Type 70 Ti Fixed-Angle Titanium Rotor	BECKMAN COULTER	337922
Ultra-Clear Tube, 25 x 89 mm	BECKMAN COULTER	344058

Comments/Description

Standard PCR
Standard PCR
Control the cells during the microaspiration process
Culture
Culture
Culture
Observation/Monitoring
Observation/Monitoring
DNA extraction
Cell count
Standard PCR
Microaspiration and release of cells
Microcapillary positioning
Standard PCR
Virus purification
Culture/observation
Filtration
Fluorescent molecular probes/ flow cytometry
Standard PCR; DNA gel stain
Electron microscopy
Virus purification
Virus purification

jove
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.3051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Single cell micro Aspiration as an alternative strategy to fluorescence-activated cell sorting for giant viruses

Author(s): D. Saliu-Bonnisau, P. VDH Boratto, G.P. Oliveira, S. Y B. Khalef, B. La Scola, J. Andreani

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	ANDREANI Julien
Department:	MEPHI Lab
Institution:	IHU Mediterranée infection
Title:	PhD
Signature:	Andreani
Date:	13/04/19

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

1. There is still commercial language in Figure 1 (InjectMan NI2, CellTram 4r); please remove if possible.

Authors : The figure 1 was modified, all commercial language were removed. Thank you.

2. Figure 3 is missing panel labels.

The figure 3 was modified. So we adapted our Figure legend. Thank you.

3. There is still some confusing language; I have highlighted some parts but the whole manuscript could use more revision.

The changes were made. Please find the new version. Thank you.

`Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Authors: Thank you for your remarks. We modified sentences and took all your advice into consideration.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Authors: OK Thank you.

3. Please provide an email address for each author.

Authors: ok we added all the email addresses under the affiliation section.

4. Please shorten the Summary to 10-50 words.

Authors: OK Thank you.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and figures and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Kovaslides, InjectMan, CellTram, Eclipse TE2000-S, EZ1, AmpliTaq Gold®, Syber Safe

Authors: The modifications were done.

Protocol:
1. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible.

Authors: ok we modified some sentences. Thank you.

2. There is a 10 pages limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Authors: we highlighted protocol sections for the video thank you.

3. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Authors: Ok we modified the section especially about the cloning and the separation. Please find the new substeps and we added a reference about this method for isolating single prokaryotic cell that we applied to isolate virus by indirect way.

Specific Protocol steps:
1. 1.2: Around how many amoebae is this?

Authors: its 1x 10^6 cells/mL.
2. 2.1: Please define TS medium and/or include in the Table of Materials.

Authors: thank you we clarified this point TS would signify starvation medium we added too in the table.
3. 2.3: Around how long does this take?

Authors: We added time estimation in the discussion section

.
4. 3: This is slightly confusing-when is virus incubated with amoebae? What volume is used?`

Authors: Yes 100 µL of the virus mixture are infecting the amoeba. We modified the sentences

5. 5.3: Please provide the PCR conditions you used.

Authors: We added PCR steps thank you
6. 7.1: Please provide more information on preparing the glow-dischared grid.

Authors: The glow-discharge allows to obtain a hydrophilic grid. We detailed more of this in the manuscript it's a specific technic used by the electron microscopy platform. And we showed that in the reference (Bou Khalil et al.) and in the movie that we did for JOVE previously.

7. 8: Please provide a reference for this procedure.

Thank you for all your remarks. The modifications were done.

Figures:
1. If necessary, please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Authors: This picture is an original figure made by Dehia Sahmi-Bounsiar inspired from the picture of the workstation took in the laboratory 1 A. We contacted the commercial and he said that for a schematic figure 1C it's not necessary to add a citation.

2. Figure 2A: Please provide a scale bar here.

Authors: scale bar was added thank you please find the new one.

Figure 3: Please define 'PC' and 'NC'.

Authors: PC and NC are for positive and negative controls we modified the file.
4. Please remove the embedded tables from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Thank you for all your remarks. The modifications were done.

References:
1. Please ensure references have a consistent format.
Authors: we used JOVE format. Please find modifications that we did for reference section.
Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Authors: Thank you. The table of materials was completed.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this manuscript, titled "Single cell micro aspiration as an alternative strategy to fluorescence-activated cell sorting for giant virus mixture separation" by Sahmi-Bounsiar et al, authors describe a single cell micro aspiration method for the separation of amoeba infected by giant viruses as an independent method.
The field of giant viruses face many technical challenges right from isolation of virus to its

characterization. The aspiration method described in this study appears to be simple and can be easily followed and will be a useful tool for people working in this area.
Method is detailed sufficiently for anyone to replicate.

Major Concerns:
None

Minor Concerns:
Just wondering how does the cell morphology affect the micro-aspiration process?

Authors: Cellular morphology affects microaspiration in the sense of the diameter of the microcapillary used: the cell size according to the inner diameter of microcapillary, the capacity of the membrane to deform can help the entry of the cell into the microcapillary, the adhesion of the membrane on the surfaces prevents the cells from being aspirated by microcapillary, the motility of the cells can disturb the smooth progress of the microaspiration.

Herein amoeba became rounded by the infection so it's more easy in this case to capture by the capillary.

There are grammatical errors throughout the manuscript. Language is not always easy to follow. It appears that the "instructions" (protocol) have been combined with the "findings" (of the method development) that is leading to some confusion. Manuscript needs to be read for English and some parts have to be rewritten.

Authors: we modified and improved all sections. Thank you for all your constructive remarks, we took note and have made the necessary changes

Reviewer #2:
Manuscript Summary:
This manuscript describe a protocol to sort single virus-infected amoeba cells using microscopy inspection and micro-aspiration to achieve clonal virus isolates for further characterization and investigation. Overall this is an interesting technique that has the potential for significantly advancing the discovery and characterization of giant viruses, but also of other viruses of protists.
That said I strongly recommend the authors carefully revise the manuscript to improve the grammar and word choices. I found myself too many times second guessing what it was meant with specific sentences and re-phrasing sentences in my head to try to get to the point (I think) the authors were trying to convey. I do not think there are major flaws with this interesting method, just the (significant) editorial corrections are needed.

Major Concerns:
- Significant editorial editing is needed to allow readability and flow. I am guessing the video will help understanding the workflow. For example, I am very confused why step 2 is necessary rather than doing directly step 3.

Thank you for all your remarks, we took note and made changes.

We think that we needed to infect the amoeba before applying the micro aspiration so we believed that we needed to conserve this step 2 in the video.

- It is not clear to me why flow cytometry sorting did not work to get clonal viruses in the end. Maybe I got lost in translation and the point was made. In that case, it needs to be clarified by improving the grammar and writing style (including word choices).

Do single amoeba cells get co-infected by multiple viruses? Or do the authors mean that they could not get a single infected cell without carryover free or non-specifically attached viruses? Or is it simply that they could not tell apart one cell infected by one virus type from another cell infected by a different type of virus within the same culture? In my experience and that of others, single cell sorting by flow cytometry (for cells smaller that 20-40 um) is precise and provides single cells in most of the sorted droplets.

Flow cytometry sorting is routinely used by several groups to achieve "culturable" clonal isolates as well as to produce single amplified genomes. Additionally, while not yet as extended, fluorescence activated flow cytometry sorting is also possible for sorting single viruses (from fixed and unfixed mixed assemblies) and virus-infected cells. I suggest that the authors try to take a step beyond their experience (lines 68-75: "...we were unable to observe... We failed to separate...") and make an effort to briefly describe relevant work by other groups and how the approaches compare or complement each other. And why and when one would want to use this approach; specially if working on a different system, which by the way, it is not mentioned if the method can be used beyond the amoeba-virus system. My understanding from reading this protocol is simply that it is another useful way to obtain clonal viruses, but not the only one. For example, I wonder if the authors tried to sort single unfixed viruses into healthy cultures to produce infection and propagation. Even virus populations with overlapping FCM signatures can be separated by sorting single particle

???

Authors: We modified the introduction to be clearer about what we did before to use the amoeba capture procedure. Thank you for all your comment

When we discovered that we had 2 viruses after sequencing and assembly, we tried to separate by FCM as you said by sorting single particle of virus in 96 well plate and after replication but we failed because we didn't obtain cytopathic effect and any amplifications by PCR. We totally don't know why we failed. It's not technical problem because we used sorter for other cell and virus before as we published for Cedratvirus mix with Mimivirus. So we radically changed and used amoeba capture procedure. We had no idea about nature of the unknown virus.

However, it could be possible to separate infected-amoeba. We succeeded by another method, so we added this interesting comment in the discussion section. It could be an alternative method. When we didn't possess information about the low abundance it is sometimes difficult to know what the more appropriate technic could be. Thank you again and please find our modifications in introduction and discussion sections.

- Is the choice of virus mixture just an example using available, previously isolated, viruses. Does the method work with environmental samples, has it been tested? I guess so, so a simple mention of that would help.

The mixture came from an environmental sample after the co-culture process. The new viruses were isolated for the first time. The mention is added. Thank you.

- If the Usurpaviruses and the Clandestinoviruses are so low abundance in the mixture, how many single cells is the recommended to sort to be able to isolate them among the highly abundant Faustoviruses? Without this kind of discussion is difficult for this reviewer to fully understand if this is a good method of choice to reliably isolate low-abundance clonal viruses, or if the Sahmi-Bounsiar and colleagues were just lucky. Again, maybe a high-throughput method such as fluorescence-activated flow cytometry sorting is a more time and cost-efficient method.

Authors: the question of the abundance is not so easy to answer because first of all we worked from environmental samples where we don't know the real viral abundance. Co-culture process probably amplifies the most abundant virus and the one that have the capability to multiply higher in this amoeba as a close-system. But we observed also in the lab (data unpublished) that medium used for the co-culture have an impact on the viral isolation. Indeed, with the same sample and using one medium we isolated a Pandoravirus with another medium we isolated a Mimivirus and it is not a random event. We believed that the interaction between the amoeba-Virus are modified why? We don't have the answer.

So by FCM on 96 well we failed totally as explained before by sorting viral subpopulations. However, we added this as an alternative in discussion section. Thank you

Minor Concerns:
- The term "hidden" viruses is misleading. The viruses are not hidden or disguised, they are just low-abundance. I suggest the use of "low-abundance".

Thank you for your suggestion, we have replaced hidden by low-abundance in the line 99.

- L56: please, mention briefly what those strategies are. I understand the word and space limitation but it would be very helpful to describe things succinctly instead of just providing reference numbers.

Ok we attempted to be more precise. Thank you

- L91: How was MOI calculated for each virus if they could not tell them apart? or was this a mixture created from clonal viruses to serve as example for the method?

Authors: The MOI was calculated for the mixture of viruses (Fautsovirus+Usurpativirus LCD7 or Clandestinovirus ST1). We modified the sentence. This step needs to reduce the

viral titer if it's too high we have a risk of infection by the major abundance viral population because with the co-culture process we can obtain 10^7 virus/mL in a 500 microliter well.

- L93: An indication of the time often needed for full lysis would be helpful here.

Ok, the details have been added in the line 128-129. Thank you

- L98: what was the size of the viral inoculum?

The viral inoculum was 100 microliters. The precision was added in the line 136.

- L162: I do not understand what is meant here by "positive culture samples"

Positive culture sample is the sample where we observed a cytopathic effect.

- L166-168: I suggest using the very same nomenclature for these genes here and in Table 2.

Thank you for your suggestion. We made the necessary modifications.

- L169-173: please provide the thermocycling conditions.

The conditions were added in the 228-231. Thank you.

- L201-202: What are those steps? Mention and provide useful references please. Are the characterization steps for giant viruses different than from any other virus?

Authors: The steps contain the genome sequencing, the genome assembly and bioinformatics analyses and study of their replicative cycle. We added references thank you

- L204: The description of representative results is too vague. I suggest adding some more details. Also, the use of the term "cloned" is confusing. Do the authors mean "clonal" or that the genome or some DNA fragments were "cloned" into a plasmid (molecular biology tools)...

Authors: We developed our first results more, however, about genome sequencing and other studies they are ongoing in the laboratory.

-We mean clonal; the correction was done in the line 305. Thank you

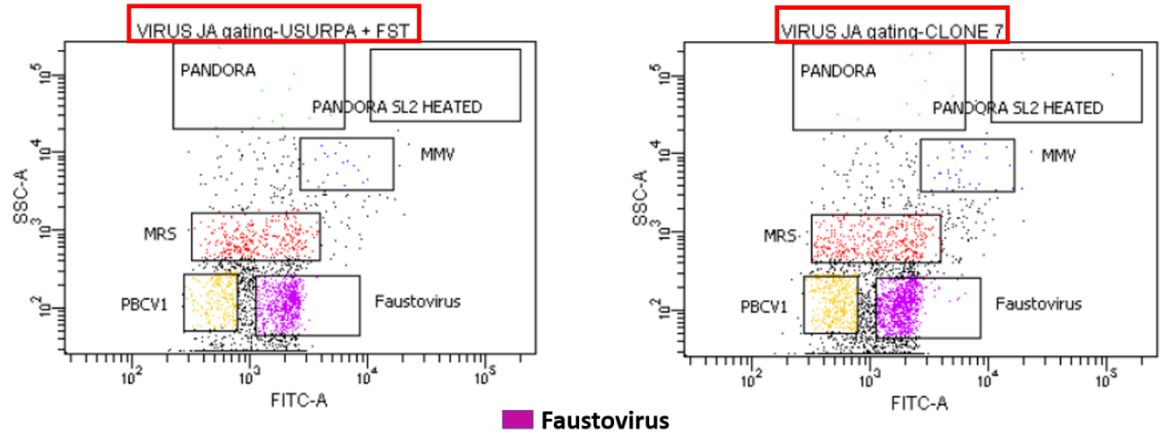
- L233: Figure 5 is not referred to in the text. Where the virus samples fixed prior to staining with SYBR Green (also, please indicate which SYBR Green was used). What is the difference between the 5 Faustovirus strains (please indicate which strains these are) on the left hand

side and the Faustovirus ST1 on the right hand side? Do they have different genome size, capsid size, and/or capsid morphology

Authors: We added it. We used Sybr green type I and added it in the table section. They don't have any difference about morphologies or genome size between Faustovirus ST1 and other Faustovirus we currently don't know. It will be something to elucidate in furthers studies.

It would have been useful to show a flow cytometry dotplot of Usurpaviruses too for comparison.

Authors : we modified the figure 5. gates are given as indicatives. We obtained the same superpositions between sub-populations of Faustovirus LCD7 and Usurpativirus LCD7.



Dark and light green and probably more descriptive than "dense" and "bright" green.
The figure key only shows two shades of purple for Clandestinovirus ST1 and for Faustovirus. Either show a full key with all colours and virus types or remove the key and leave only colour description in the figure legend.

Thank you for your suggestion. We made the necessary modifications in the line 344 and with the modification of the figure key.