

An instrumental and computational pipeline for visualizing free viral particles

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ABSTRACT

We are presenting the application of an optical and computational pipeline FAMOUS for revealing the presence of free viral particles named “virions”. The idea of such a protocol is to give rise to images of virions in their environment with a soft solution for recording the native image, contrary to the standard solution of imaging virions with electron microscopy (EM) for visualizing viral particles. The final aim of the current work is to observe free viral particles of SARS-CoV-2, the virions responsible for the worldwide pandemic of Covid-19. But such particles have diameters between 80 and 120 nm, a dimension smaller than the resolution limit of optical-only microscopy solutions. We have chosen to start with the biggest free virions, cytomegalovirus (CMV), a virus from the herpesvirus family also named “Human Herpes Virus 5”. Two kinds of cultures were involved: a fluorescent culture (BAD) and a label-free one (VHLE), both being collected from infected cell culture. VHLE virions were first observed after secondary immunostaining and concentrated with magnetic nanoparticles and then without labelling. The optical protocol rests on a standard solution of multiphoton microscopy combined with a computational strategy based on the point-spread-function (PSF) recordings, its mathematical modeling and the restauration of the image resting on the PSF model. A test with free viral particles of SARS-CoV-2 is led, delivering an optical visualization of the free-viral particles. The visualization of objects aggregates obtained in both situations confirm the relevance of the pipeline FAMOUS for imaging free virions.

Keywords: Optical imaging of virions, Cytomegalovirus, Multiphoton microscopy, Pipeline FAMOUS, image restauration

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1. INTRODUCTION

The understanding of viral infection is a current topic of first interest which as gained in scientific attention with the international health pandemic of Covid-19 [1]. Thus, scientists from numerous fields have started to work with virologists in worldwide public health: chemists, biologists, economists, physicists, humanists and computer scientists [2-5]. The final goal of the project CoviZion is to provide a better representation of the SARS-CoV-2 virus and its biological modes of action to better understand societal representations of the pandemic. Indeed, the links between scientific images of viruses and what they generate in terms of social imagination provoke reasonable or unreasonable fear, suspicion or distrust from society towards the scientific and medical communities. Does the visualization of the invisible enemy contribute to a better understanding of the pandemic? The question of the societal mode of action of a virus on a population requires to understand simultaneously the biological mode of action of this public enemy.

The question of the way of action of the virions into the host cells is of first interest. The overall process is fairly well known. First, the viral proteins present on the surface of the virion will bind to the specific receptors of the targeted cell, inducing the formation of vesicles during the internalization of the viral particle. The fusion of the membrane vesicle with a lysosome will induce the fusion of the viral envelope with the lysosomal vesicle, thus releasing the viral genome, in this case RNA, into the cell. The RNA sequence will then be translated by the ribosomes of the cell, inducing the synthesis of the proteins constituting the viral particles as well as the RNA polymerase, responsible for the synthesis of copies of the viral genome. The different parts of the virus will then assemble into virions, creating new army of viral

particles which will be transported outside the cell via vesicles. These viral particles will thus be able to infect new healthy cells by repeating the same process [6].

A current question in the virology community concerns the specifics of the detailed mode of action of virions during colonization. Indeed, if all viruses follow the mode of action described briefly above, each virus follows different pathways to leading them to the replication and the release steps. For example, during the first step where virions attach to the cell wall, different membrane proteins are involved on the virion side and on the cell wall. Now, we very well know that S-proteins play a key role in the infection with SARS-CoV-2 virus [7]. But S-protein are specific to SARS-CoV-2 and are not necessarily present for other viruses. The second question concerns the gateway of entrance of virions into the cell. What are the membrane proteins involved? Thereafter, how does the viral particle reach the energy reserve and the genomic area of the cell in order to process the replication steps? What are the biological components of the cells involved into this process?

The idea of this current research work, started in the middle of the year 2020, is to benefit from two technical solutions already existing in the panel of instrumental solutions devoted to imaging targets coming from life sciences. But the free viral particles of SARS-CoV-2 have diameters between 80 and 120 nm [8], a dimension smaller than the resolution limit of optical-only microscopy solutions, about 300 nm in routine use [9]. A process of imaging with super resolution strategy should have been interesting, but the free viral particles are vulnerable objects regarding to the laser parameters required in this kind of solution. By consequence, this question could be asked in the context of current solution existing in biology, already implemented in optical microscopy for biology. The first one is the immunochemistry, resting on the specific labelling of proteins, with a selectivity adequate with the selection of proteins chosen [10]. This solution is already well mastered by the specialists of immunochemistry and biology. The second, an instrumental solution, rests on optical microscopy, which is the exclusive solution of optical characterization able to produce images of living samples without a drastic protocol of labelling, or a drastic protocol of experimentation such as in electron microscopy with extreme conditions in pressure and temperature. We have recently demonstrated the high interest of merging an instrumental solution of optical characterization, the multiphoton microscopy (MPM), with the most recent computational solution for image restoration resting on the estimation of the Point-Spread-Function (PSF) of the instrument, its mathematical modeling, and the image restoration considering the instrumental response function mathematically described all along the 3D view point of the image. The technical details of the instrumental and computational pipeline FAMOUS have been published in 2021 in [11]. But the question of the object dimensions remains unsettled. The optimization of the image resolution thanks to numerical solutions is a complex strategy that have to be addressed progressively. Thus, we have selected objects in the scientific field of virology, with the biggest known dimensions.

In the current publication, we are testing the instrumental and computational pipeline FAMOUS for revealing the image of free viral particles named “virions”. Among the whole of the viral particles existing all around the world, many sizes exist, from 20 nm to 300 nm in diameter. And among the biggest free virions known, cytomegaloviruses (CMV) win the medal with their dimensions ranging between 150 and 300 nm. These dimensions are very close to the optical resolution, with an approximated value of 250 nm in ideal et optimal experimental conditions. Thus, we are testing our pipeline FAMOUS for delivering images of free virions of CMV. In a last trial, we are testing with the same protocol the images of free virions of SARS-CoV-2.

2. EXPERIMENTAL PROTOCOL

2.1 Culture of CMV, magnetic microspheres and labelling solutions

The cytomegaloviruses (CMV) were cultured from infected cells in cell culture medium. After 3 days of infections [12], the solutions were clarified and then stored at -80°C. Cell debris was separated from the supernatant by centrifugation. The supernatant was removed and then passed to UV for 30 sec to inactivate the viral particles. The medium was then filtered by centrifugation in 4mL Amicon tubes for 10 min at 5000 g. The filtrate was collected (200 µL) then transferred into 2 mL Eppendorf tubes and diluted by half in PBS. The viral particles were then filtered via beads on a magnetic rack the magnetic beads are constituted by a magnetic core labeled with a fluorophore emitting a signal centered at 580 nm. 40 µL of magnetic beads were added to the virion solution for incubation for 10 minutes at room temperature and 30

minutes on a magnetic rack at room temperature. The magnetic beads were rinsed twice with PBS or H₂O on a rack and then suspended in 100 μ L of PBS or H₂O. 10 μ L of the resulting solution containing the free virions were deposited between slide and coverslip for observation under the microscope. The VHLE virions were observed after double immunostaining after fixation on beads. The VHLE virions were incubated in an oven at 37°C with anti-cytomegalovirus antibodies (Ab gB) diluted to 1/40th for 30 minutes without a magnetic rack and 30 minutes at room temperature on a magnetic rack, then rinsed three times in PBS. The virions were then incubated away from light at 37°C with the anti-mouse antibodies, labeled with fluorescein, diluted to 1/100th for 30 minutes without a magnetic rack and 30 minutes at 37°C on a magnetic rack then rinsed three times with PBS. The virions were then resuspended in 100 μ L of PBS. The purpose of these different markings is to find the viruses more easily under the microscope and thus have an idea of their shape/distribution on the slide and then compare the results with the samples without fluorescent labelling.

2.2 Multiphoton microscopy and computational solution

A standard solution of multiphoton microscopy (MPM) has been used. The non-linear optical technology used for the multiphoton laser allows us to excite the focal plan, a specific part contained into the volume of images, reducing the Airy profile as well as the background noise comparatively to more standard solutions of confocal microscopy. The infrared range of excitation light involved in MPM limit the risks of damages inflicted onto the living samples such as quenching or photobleaching [13, 14]. These properties make the MPM solution a very interesting tool for the study of endogenous fluorescence. A 2D scanning device guides the laser beam onto the sample and scans it following a matrix pattern. Thus, a 2D image can be produced. The samples were observed under an Olympus multiphoton microscope (BX61WI) coupled to a sapphire titanium laser source delivering pulses in femtoseconds (Chameleon Ultra II, Coherent Inc.). The average power used for imaging the free virions equals 10 mW. Two water-immersion microscope objectives were used, according to specific properties. Table 1 lists their optical properties.

Table 1. Specificities of microscope objectives involved.

	Olympus XLPLN	Olympus LUMFL N
Reference	XLPLN25XWMP2	LUMFLN60XW
Numerical Aperture	1.05	1.10
Magnification	25x	60x
Working distance	2 mm	1.5 mm

The detection part of the microscope is composed by two photomultiplier tubes (PMT). A filter-set allows the observation of two distinct signals emission provided a distinct separation in wavelength emission ranges. Tables 2 resumes the filters involved in this set of experiment.

Table 2. Filter-set for the detection of fluorescence emission from free virions.

Primary dichroic filter	650 nm	
	Filter-set n°1	Filter-set n°2
Secondary dichroic filter	518 nm	450 nm
Transmission filter	575 – 630 nm	495 – 540 nm
Reflection filter	420 – 500 nm	260 – 340 nm

Three-dimensional image acquisitions were allowed thanks to an axial movement of the microscope objective; the precision of the axial displacement of the objective equals 10 nm, at best, allowing a scanning thickness suited to the

dimensions of the virions. Thus, 3D images can be produced with an incremental range from 10 nm to several micrometers, with a minimum step of 10 nm. We have registered images with an axial step of 100 nm between each image along the optical axis (Z axis). Acquisitions of 3D images of fluorescent microbeads of known size were carried out beforehand to calibrate the deconvolution system, using the same acquisition parameters than the ones to acquire the viral particles. The calibration step lies in an estimation and modeling of the instrumental PSF thanks to the FIGARO algorithm [15]. This PSF model is then used to restore the viral particles images, through a regularized deconvolution step within the pipeline FAMOUS, based on BD3MG algorithm [16]

3. MULTIPHOTON IMAGES OF FREE VIRAL PARTICLES

The first step of the pipeline FAMOUS concerns the identification of the sample of interest. Here, the final objective of our project CoviZion concerns the imaging of free viral particles from SARS-CoV-2. Their diameter, at best three times smaller than the optical resolution does not allow to a multiphoton microscope to image them directly. That is why we have chosen the biggest existing free virions known: the cytomegaloviruses (CMV). Their diameters are between 150 and 300 nm, a value in the range of the resolution of multiphoton microscope (optical plane).

3.1 Initial test: the ability of MPM to deliver an image of free viral particles

The first set of experiment concerns the test of the ability of MPM to deliver an image of the free viral particles. Figures 1A and 1B illustrate the raw images of CMV recorded for a sample resulting from the protocol described into the last presentation, without the use of magnetic spheres.

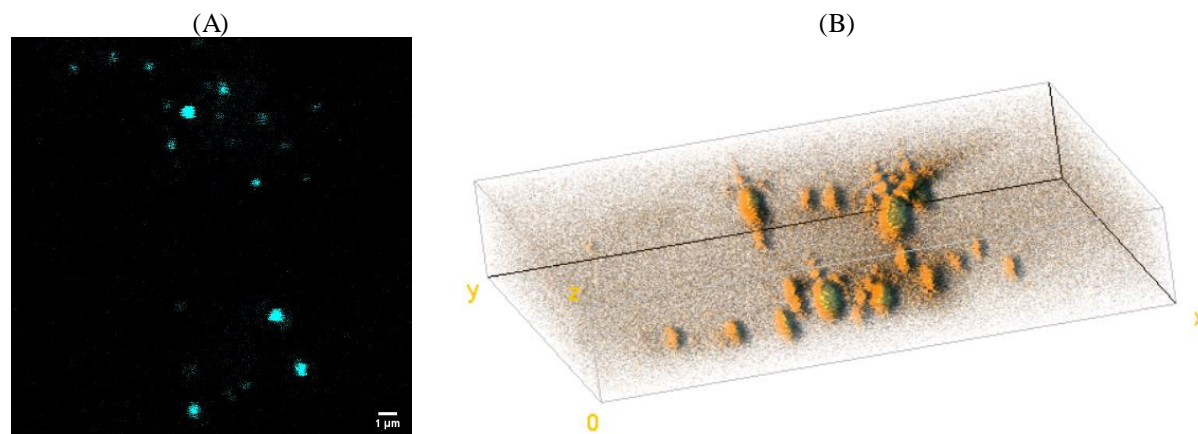


Figure 1. Raw acquisitions of CMV recorded by multiphoton microscopy. No magnetic microspheres were involved for these images. The spheroidal objects are spread all along the volume image. A. 2D representation of the imaged microscope slice. B. a 3D representation of the CMV deposited onto a microscope slide.

The object shapes look how expected: a spheroidal object with different diameters. Their relative position, especially considering the vertical organization is random, due to the solution containing the free virions which has been deposited between the microscope slide and coverslip. The difficulty observed here concerns the ability of the system to deliver an image with a resolution performance adapted to the dimensions of the CMV. This justify our second set of experiment, involving magnetic microspheres allowing to concentrate the free viral particles, that we present hereafter.

3.2 CMV concentrated with magnetic microspheres

The magnetic microspheres were included into the biological protocol of culture of free viral particles of CMV. Such a solution gathers viral particles together in packages, including all the dimensions possible between 150 and 300 nm into a single bloc delivering a signal concentrated in a specific region of the image. Thus, the computational solution is better adapted, thanks to a smallest level of background noise, a deleterious effect on the accuracy of the computer reconstruction. Figure 2 illustrates the raw recordings and the images resulting from the run of the pipeline FAMOUS. A constant average PSF have been taken into consideration for this run, taking into account that the PSF variation initially highlighted into [11] were observed exclusively into thick biological samples along the optical axis. Here the thickness of the sample is about few microns, not deep enough for producing a variation of the PSF into the optical axis.

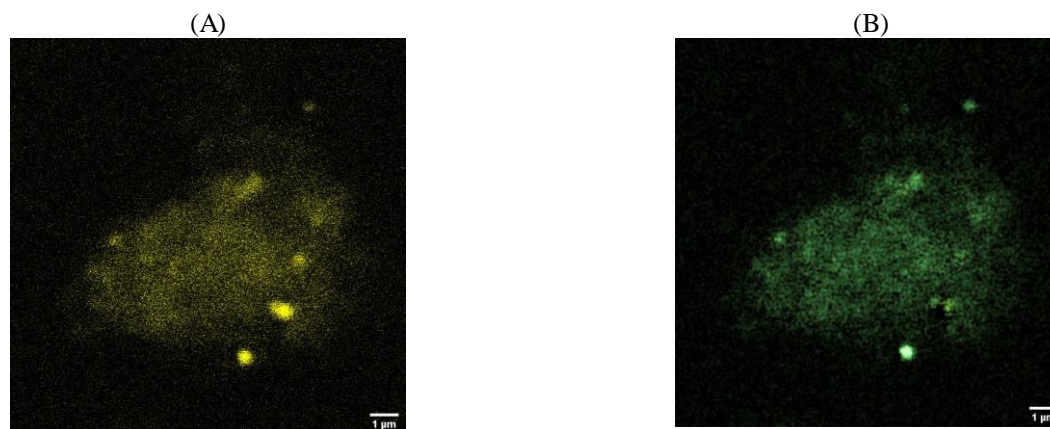


Figure 2. Images of CMV loaded with magnetic microspheres. (A) raw images. (B) Restored image with the pipeline FAMOUS, considering a constant PSF all along the imaging depth.

The interest of microsphere for revealing the image of free viral particles rests here on their ability to concentrate the free viral particle into a small volume of the microscope slide. Thus, the combined emission of fluorescent signal coming from the whole of the particle imposes a mass phenomenon, delivering a higher level of signal. Thus, a reduced average power of the excitation beam can be used, in association with a reduced level of damage. A 3D recording is thus simpler and less confronted to the virion destruction, often observed in the beginning of the experiment. Figure 3 illustrates a 3D representation of the images presented in 2D in Figure 2.

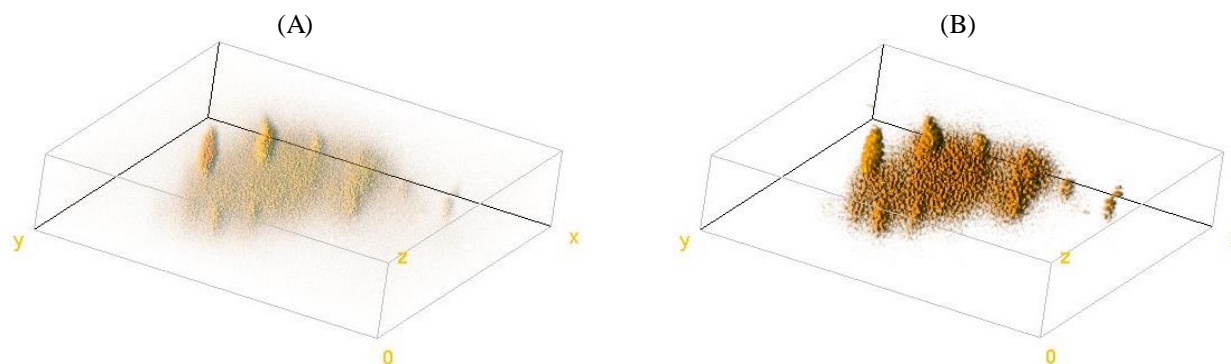


Figure 3. 3D representation of free viral particles of CMV concentrated with magnetic microspheres. (A) raw acquisition. (B) restored image produced with the pipeline FAMOUS.

3.3 Free particles of SARS-Cov-2

We have finally tested the imaging of virions from SARS-Cov-2 thanks to a similar protocol as the one involved with CMV. No magnetic microspheres were involved for this set of experiment. Figure 4 illustrates the raw recorded image.

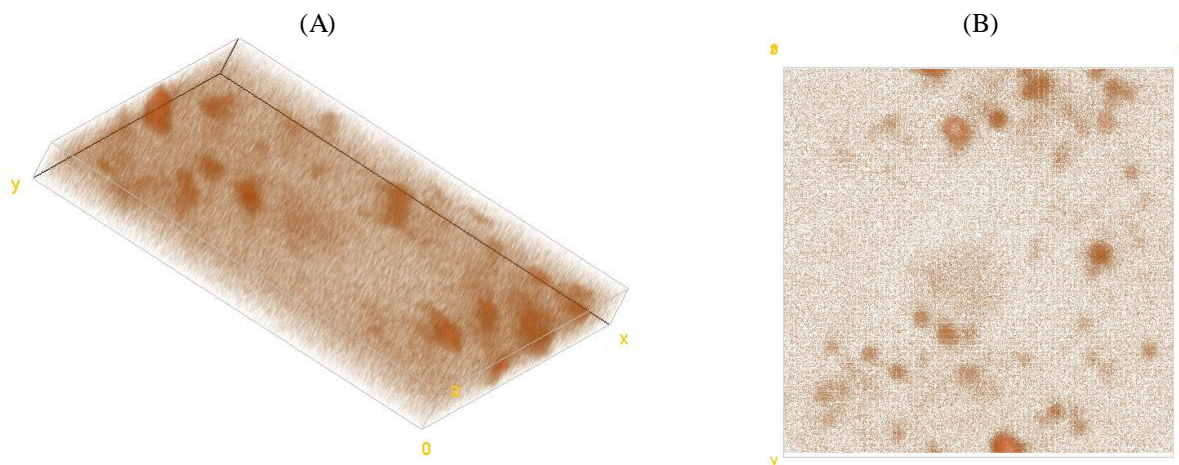


Figure 4. Raw recordings of multiphoton images of SARS-Cov-2. A. A 3D representation of the free virions; box dimensions: $15 \mu\text{m} \times 15 \mu\text{m} \times 1 \mu\text{m}$. B. a 2D plan representing the position of the free viral particles.

4. DISCUSSION ABOUT THE MULTIPHOTON ABILITY TO IMAGE FREE VIRAL PARTICLES

This first set of experiment about multiphoton microscopy devoted to the imaging of free viral particles has delivered an interesting result. Indeed, thanks to an attentive and a careful virion culture protocol, associated with numerous filtering and cleaning steps to keep only individual particles, the MPM solution delivers images of free viral particles. This result was not initially expected due to the dimensions of these objects, smaller than the resolution of the microscope. But, free viral particles are not necessarily individual objects as they can form groups of particles delivering an emission signal level higher than the individual virions, thus delivering an exploitable image. In the present work, images of virions from CMV and SARS-CoV-2 are revealed. The images presented here are coming from labeled particles, an easier way to obtain exploitable signal, able to be numerically restored with our pipeline FAMOUS.

We have also tested the same protocol thanks to free viral particles of CMV and SARS-Cov-2 without any labelling process. A signal was detected among a higher level of background signal. The emission beam from the virions exists and is possible to detect, regardless of a significantly degraded signal-to-noise ratio. But the computational solution is not enough robust to noise to identify isolated free viral particles as it is the case for labelled particles as presented in Figures 1A, 1B, 2B and 3B.

The origin of the detected signal could be discussed. Only hypotheses can be delivered here. Indeed, CMV is a virion with a double-stranded DNA while SARS-Cov-2 is a virion with a single-stranded RNA with positive polarity. DNA and RNA are substances composed by an assembly of nucleic acids. These substances are composed by chemical components able to generate a signal of fluorescence providing an adapted excitation solution, here with a two-photon process, and an adapted wavelength range of detection.

5. CONCLUSION ABOUT THE INTEREST OF OPTICAL IMAGING FOR REVEALING FREE VIRAL PARTICLES

The aim of the current experiments is to propose a solution for visualizing the free viral particles of cytomegaloviruses and SARS-CoV-2 virions, thanks to an optical solution of microscopy. The initial results presented here have shown an interesting point: the free viral particles can be observed through an optical microscope. By evidence, such a solution will not give any details about the internal or external structuration of the particle by itself. The interest of the optical imaging of the free viral particle rests on its ability to deliver in live information coming from living samples operating in physiological conditions. Indeed, among all existing technical solutions to provide evidence on biological targets under specific experimental conditions (influence of a marker, a molecule, object locations...), optical microscopy is the exclusive solution allowing to observe phenomenon without drastic experimental condition (temperature, pressure, labelling strategy, laser average power or beam shapes...). Thus, the first step highlighted here is the ability of an optical microscopy strategy to reveal the presence of biological objects having dimensions close to the resolution limit. In our case, this first step is ready; we went a little further in the test thanks to the demonstration of the ability of our original computational strategy associated to multiphoton microscopy to deliver restored image thus showing an improved visual quality of the image of the biological object. For the future, the next step could be to record a sequence of viral infection of cells under a multiphoton microscope and to observe the viral pathway to propose new solutions for blocking the viral infections.

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