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Observation of SARS-CoV-2 viral particles using electron microscopy-guided multiphoton microscopy

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ABSTRACT

We tested the ability of multiphoton microscopy (MPM) devices to produce label-free images of the virus Sars-CoV-2 responsible for the Covid global health crisis of the years 2020-2022. The virion cultures were grown in our high-security virology laboratory and then optically inactivated so as to preserve the integrity of the virus without its contagious nature. We used magnetic beads for concentrating the particles and increasing the viral quantity. We have determined a spectral range of excitation and emission, combined with adapted energy levels for the non-destructive excitation and efficient collection. We obtained a discriminating signature of these viral particles thanks to the nonlinear optical process involved in MPM and produced an image of virion bundles concentrated around magnetic particles. We have confirmed the origin of the MPM images from the SARS-CoV-2 viral particles thanks to electron microscopy images. This superresolution imaging solution has allowed to reveal the shape of viral particles of SARS-CoV-2 in their entirety, combined in clusters around magnetic particles, the core of which is clearly identified by its diameter of 200 nm. We attributed the origin of the optical signature from the virions to the presence of a large quantity of genetic material in the free viral particle. This substance, the DNA or RNA, is made up of an assembly of nucleic acids with a known nonlinear optical property. We are confident that the assembly of these formally non-linear elements enabled the creation of the multiphoton microscopy signal.

Keywords: Multiphoton microscopy, SARS-CoV-2, Label-free imaging, Electron microscopy

1. INTRODUCTION

The specific biochemical processes that result in viral infections are unique to both the viruses and the host cells involved. What are the key viral proteins responsible for the viral infection? Which cellular receptors are involved? So many questions that can sometimes be answered by specific biochemical studies [1]. Microscopy solutions have been seldom used to provide answers to these questions. Indeed, virions, the free viral particles, have dimensions smaller than diffraction limit, making them invisible with optical microscopes [2]. The experimental conditions of alternative solutions of superresolution approaches are not fully adapted for a live visualization of viral particles, requiring a complex optical setup [3]. Environmental scanning electron microscopy (ESEM) is a solution of superresolution imaging delivering a 2-dimensional (2D) images of fixed biological objects. But no in live visualization of biological phenomena is possible with ESEM. Only optical microscopy allows such an in live observation at the submicrometric scale. Nevertheless, objects of a hundred nanometers in diameter have dimensions 3 times smaller than the diffraction limit. Optical microscopy plays a crucial role in imaging viral particles, providing valuable insights into their interactions with their environment. However, superresolution optical microscopy solutions are incompatible today with the imaging of fragile particles like virions or for revealing in live processes.

In this context, it is necessary to strike a balance between obtaining super-resolution information and capturing real-time, live data. Multiphoton microscopy (MPM) is an advanced imaging technique that utilizes nonlinear processes of two- or three-photon fluorescence and/or second harmonic generation [4]. MPM presents the ability to capture detailed but diffraction-limited images of live specimens with minimal damage and sometime label-free. MPM has a label-free

imaging capability, which is a significant advantage for biomedical applications [5]. MPM can visualize and study biological structures and processes without the need for external contrast agents or fluorescent labels. In some specific cases, the use of an optical fiber can deliver the excitation signal into a localization difficult to reach, giving to this technical solution a flexibility particularly interesting [6]. When the image quality is not enough to deliver interpretable information, a numerical strategy is used and can optimize the image quality by the simultaneous recording of the image and the point spread function [7].

The intrinsic emission of light by certain molecules or structures naturally present in biological tissues or samples is a phenomenon highly known from users of optical microscopes. This endogenous fluorescence often comes with certain challenges and potential negative effects. Indeed, endogenous fluorophores often exhibit broad-spectrum fluorescence, making it challenging to achieve high specificity in imaging [8]. This limitation can hinder the ability to distinguish between different cellular components solely based on their endogenous fluorescence. This background signal can interfere with the detection of specific fluorescence signals, reducing the signal-to-noise ratio and the overall quality of imaging. Conversely, the endogenous fluorescence of living tissues is sometimes considered as a powerful tool for studying cellular and tissue biology, providing valuable information about metabolism, structure, and function in a non-invasive manner. Common endogenous fluorophores include metabolic indicators (NADH or FAD), collagen, elastin, keratin, myosin, porphyrins...

The optical characterization of free viral particles such as SARS-CoV-2, the virus responsible for the Covid global health crisis of the years 2020-2022 has been reported in 2021 thanks to a nice approach of superresolution imaging combining an ultrasensitive interferometric method with deep learning [9]. We have recently observed the fluorescence emission of free viral particles and bacteria thanks to endogenous multiphoton fluorescence. At that time, we were able to reconstruct an optical image of aggregates of free-viral particles with MPM, including SARS-CoV-2 virions [8]. The comparison with cytomegalovirus (CMV), an herpesvirus responsible for congenital infection was led thanks to the high dimension of these viral particles of about 300 μm .

In the current publication, we are proposing a complement of these results in order to show a complementary illustration of the optical images produced. Indeed, the optical images has been obtained after a first set of visual characterization with ESEM. These images have revealed visually the presence and the quantity of free viral particles of SARS-CoV-2 and their organization regarding to the culture protocol involved.

2. EXPERIMENTAL SETUP: VIRAL PARTICLES AND MULTIPHOTON MICROSCOPE

2.1 Preparation of free viral particles

SARS-CoV-2 viral particles were collected from infected cell culture media and were stored at -80°C. The supernatant was clarified by centrifugation to remove cell debris and then passed under UV light to inactivate the viral particles by breaking the RNA bonds in their genetic material, removing their infectious potential. The medium was then filtered by centrifugation in Amicon 4 mL tubes (10 min at 5000 g) to concentrate the virions; 200 μL of virion suspension was taken then transferred into 2 mL Eppendorf tubes and diluted by half in PBS; 40 μL of magnetic beads were added to the virion solution to filter the viral particles (10 min of incubation and 30 min of incubation on a magnetic rack at room temperature). The magnetic beads were rinsed twice with H₂O or PBS on the rack then suspended in 100 μL with H₂O or PBS. Each slide used for microscope observations contains 10 μL from these solutions. A similar protocol of preparation was led for CMV. Beads without virions were prepared in parallel as a negative control.

Viral cultures were performed at the virology laboratory of the University Hospital of Limoges France. SARS-CoV-2 strain variant 20A was obtained from intranasal samples from an infected patient, inoculated in VERO cells (ATCC® CRL1586™) and subcultured to produce cell-free virus stocks in the high security laboratory (P3). Samely, CMV strain AD169 (ATCC VR-538) was cultivated in embryonic human fibroblasts (RDBiotech) and subcultured to obtain cell-free virus stocks.

2.2 Optical setup for label-free fluorescence imaging

The optical setup was a standard multiphoton device with a mode-locked titanium-doped sapphire laser system (Ti: Sa). Femtosecond pulses were delivered in the near infrared range between 680 and 1080 nm (Chameleon Ultra II, Coherent). The detection ranges are located in the visible range where two separated channels can record simultaneously two emission ranges and reconstruct an image of the sample for each of these wavelengths. An Olympus commercial multiphoton microscope (model BX61WI) was utilized for the entire setup. The multiphoton images were managed with the free software Image J.

2.3 Environmental scanning electron microscopy for visualizing viral particles

The environmental scanning electron microscope (ESEM), FEI ESEM Quanta 450 FEG, was used to visualize a specific view of the surface of the viral particles at higher magnification [10]. This technique allows visualization of samples at the nanometer scale and offers direct imaging of viruses utilized for research purposes. At IRCER laboratory, it became possible to imagine viruses without causing shrinkage, ice crystal formation, or distortion of the biological sample. This was achievable through ESEM, allowing the sample to remain in a normal hydrated state within minutes of introduction into the analyzing chamber. The scanning electron microscope was used with the following characteristics: pressure between 500 and 650 Pa, voltage between 5 and 10 kV, mag 30000-50000, humidity of the chamber between 70% and 95%.

3. VALIDATION OF MPM IMAGES OF VIRUSES CONTENT WITH ESEM

The goal of this section is to show in the left-hand column the ESEM images of free viral particles and in the right-hand its corresponding image in MPM and the corresponding spectral ranges of detection. Figure 1 gathers these images from each of the imaging solution.

The ESEM solution is by evidence an optimal solution when the observation of viral particles in their entirety is required. We can count the quantity of particles and evaluate their maximum and minimum diameters.

In the images presented in Figure 1, maximum diameters of viral particles are about 0,23 μm for SARS-CoV-2 and 0,64 μm for CMV. In the images presented in Figure 1, minimum diameters of viral particles are about 0,07 μm for Sars-CoV-2 and 0,12 μm for CMV. The dimensions observed are slightly bigger than the expected dimensions. Indeed, the viral particles of SARS-CoV-2, which are devoid of the virus, typically range in diameter from 80 to 120 nm and in the range of 150 and 300 nm for CMV [11-13]. By evidence, even with ESEM, the smallest particles are not visible, even though they are probably the most numerous. The ESEM or optical microscopy solutions are not adapted for measuring the particles dimensions in the case of viral particles.

The consequence of the measurements given by the ESEM images is that we understand why the images of MPM give the impression of showing a much smaller number of particles than the samples actually contain. Thanks to the ESEM images, we can determine that the dimensions of the viral particles are in the range of the optical resolution of the MPM device, for both Sars-CoV-2 and CMV free viral particles. By consequence two kind of objects can be visualized at the optical image for both Sars-CoV-2 and CMV free viral particles:

- the individual viral particles which have a diameter bigger than the optical resolution estimated at 200 nm
- a cluster of particles which have a diameter bigger than the optical resolution. Their emission signals are cumulated and the movement of the package of free viral particles can be followed.

The last point is interesting for following the path of free viral particles in an infectious situation. The MPM images can thus visualize both the individual viral particles which have a diameter bigger than the optical resolution and a cluster of particles.

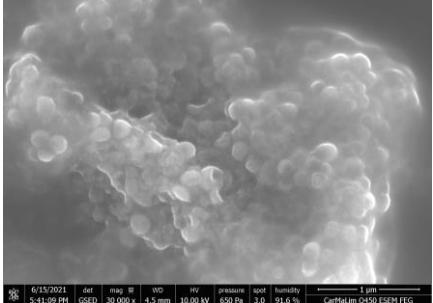
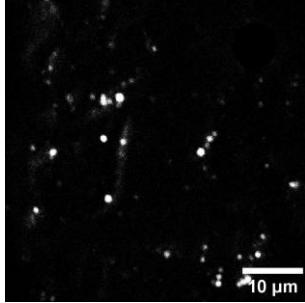
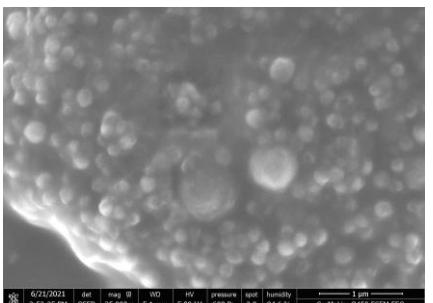
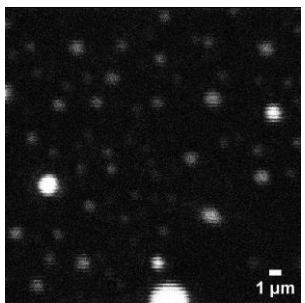
	ESEM	MPM	Measured diameters	Spectral ranges of excitation and detection
Sars-Cov-2			Maximum diameter: 230 nm	Spectral range of excitation 900 nm
			Minimum diameter: 66 nm	Spectral range of emission 420 – 500 nm
CMV			Maximum diameter: 640 nm	Spectral range of excitation 750 nm
			Minimum diameter: 120 nm	Spectral range of emission 420 – 500 nm

Figure 1. Illustrations of the observation of SARS-CoV-2 and CMV viral particles using electron microscopy-guided multiphoton microscopy. The maximum and the minimum diameters of the free viral particles are estimated with the ESEM images. The spectral ranges of excitation and of detection in MPM are reminded.

4. CONCLUSION

We used Environmental Scanning Electron Microscopy (ESEM) to visualize viral particles with a different method from optical microscopy for a better visualization of their surface as well as a higher resolution to confirm the presence and shape of virion clusters. The magnetic beads observed with the scanning electron microscope are visibly different in terms of their surface, which is rougher and more raised, and in their composition. The viral particles have a dark marking on their envelope as well as a smoother surface. The shape of the viral particles and their size seem to correspond to the expected scales and the prolonged zooms at high pressure by the microscope induced the destruction of the envelopes in a few seconds. These images allowed a better understanding of multiphoton microscopy observations in the distribution of viral particles around the magnetic beads.

More generally, the images can be highly detailed with ESEM but not at all with MPM. The interest of the imaging of free viral particles in MPM stays in the ability of this solution to image living sample during the infectious process, a situation which cannot be revealed with ESEM. In this real biological situation, a viral contamination of a cell involves a high viral load, meaning that a high quantity of viral particles is present. Their whole signal can be detected and their presence around the host cell and then inside the host cell could be followed thanks to the endogenous signal produced under a multiphoton excitation.

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