Research Article

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Future VIROLOGY

Direct virus capture assay for label-free detection of SARS-CoV-2 virions using laser microscopy

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Aim: To evaluate a label-free and variant-independent assay called direct virus capture (DVC) for detection of intact SARS-CoV-2 virions through light scattering, utilizing an optical inverted laser microscope called nano eye device virus detector (NED-VD). **Methods:** The DVC assay involves the interaction between ACE2 receptors printed on a glass coverslip substrate, and the S protein on the outer surface of virions. The study was conducted using 191 human swab specimens. **Results:** In comparison to the RT-PCR assay, the DVC method achieved a sensitivity of 40.5%, specificity of 90.4% and accuracy of 46%. **Conclusion:** The study presents a promising qualitative pre-screening test to evaluate the presence of whole virions and reduce the number of PCR tests.

Plain language summary: SARS-CoV-2, the virus responsible for the COVID-19 pandemic, can spread quickly from person to person and can make people very ill. An important part of controlling the spread of the virus is to detect infections early through diagnostic tests. This study presents a new test called direct virus capture (DVC). In this test, the virus is frozen on a glass slide and visualized with a laser microscope. We tested samples from 191 patients using DVC and the most common diagnostic test for SARS-CoV-2. Both methods identified the virus in the samples, but DVC was able to capture the whole intact virus. Only in this form is the virus able to attack human cells and cause disease.

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Keywords: biosensing • intact virions • label-free • microscopy • SARS-CoV-2 • scattering

Since late 2019, an outbreak of a new severe respiratory disease in humans of unknown etiology emerged at first in Wuhan, China and quickly spread all over the world [1,2]. Subsequently, Chinese health authorities identified the causative agent of this new clinical disease as a novel coronavirus, named SARS-CoV-2, leading to the designation of the disease as COVID-19. The rapid global spread and high mortality rate prompted the WHO to declare it a pandemic on 11 March 2020 [3].

One of the primary objectives from the moment of the identification of SARS-CoV-2 has been the development of high-performance and rapid diagnostic tests to promptly identify infected individuals and mitigate further transmission [4]. Additionally, assessing infectivity and the viral shedding timeframe in SARS-CoV-2-positive individuals has become a matter of critical concern. The laboratory diagnostic strategies and techniques for detecting of SARS-CoV-2 fall into two main categories, in line with the target compounds: molecular and serological [5].

Molecular methods involve the detection of viral genetic material through nucleic acid amplification tests (NAATs) and/or genome sequencing. The availability of pathogen genomic sequences in public databases, including newly emerged viruses like SARS-CoV-2, has facilitated the rapid development of PCR kits with high specificity, sensitivity and adaptability for the detection of emerging viral variants [6]. Today, real-time reverse transcription PCR (RT-PCR) is the gold standard technique for diagnosing COVID-19, quantifying the viral load in a sample by measuring the number of viral RNA copies per milliliter [7]. RT-PCR amplifies and detects specific RNA sequences

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of SARS-CoV-2 in various forms, such as free RNA, RNA within intact virions or RNA from damaged or degraded virions [8]. However, RT-PCR does not discriminate between encapsulated viral RNA within viable and infectious virus from other forms of viral nucleic acids. The Ct value is a measure of the number of PCR cycles required to reach a specific fluorescence threshold, and inversely correlates with the viral load, with every ~3.3 Ct unit increase representing a tenfold reduction in the starting material [9].

Serological methods involve the affinity bound between viral proteins (viral antigens) and specific antibodies to assess the infection. Several viral proteins, for example the S protein in coronaviruses or the HA protein in influenza virus, act as antigen for specific antibodies [5]. Conventional methods include enzyme-linked immunosorbent assays (ELISA) or immunofluorescence staining, which offer qualitative or semi-qualitative results with adequate sensitivity [6,10]. Rapid antigen diagnostic tests (RADTs) based on immunochromatography (IC), or lateral flow assays (LFAs) have become popular due to their disposability, low cost, fast turnaround time, qualitative results and suitability for point-of-care testing. Furthermore, serological assays identify microorganisms indirectly by detecting specific antibodies generated during the immune response. These tests are useful for retrospective diagnosis, surveillance, and epidemiological studies, providing prevalence estimates and monitoring antibody levels over time to assess acquired protection or immunity [6,11].

In addition, nowadays the COVID-19 molecular and serological diagnostic tools comprise a variety of biosensors characterized by different signal conversion systems, including electrochemical, nano plasmonic, optical and others. In this regard, the optical ellipsometry-based approaches if compared with the most common SARS-CoV-2 detection assay such as Western Blot, RT-PCR, ELISA and indirect fluorescence has demonstrated to be a direct, non-destructive, rapid, label-free and low-cost test [5]. In particular, the spectroscopic ellipsometry in total internal reflection mode (TIRE) have been gained interest in antibody-antigen interaction measurements due to its higher sensitivity than SPR [12].

Before the advent of molecular and serological methods as diagnostic tools for detecting respiratory viruses, viral culture was traditionally regarded as the reference standard technique. The viral culture offers the distinct advantage of providing direct insights into viral pathogen identification, replication, and quantification. However, its significant disadvantage is its extensive turnaround time, typically spanning several days, rendering it impractical into the routine clinical workflow [4,6]. Moreover, the diagnostic application of culture is limited by technical expertise and costs. Beyond viral culture, also several advanced microscopy techniques emerged enabling the visualization of intact virions and delivering direct information about their morphology and quantitative assessment. These techniques encompass transmission electron microscopy (TEM) [13], scanning electron microscopy (SEM) [14], cryo-electron microscopy (Cryo-EM) [15], atomic force microscopy (AFM) [16] and fluorescence microscopy (FM) [17].

These methodologies are invaluable in the realm of scientific research; however, they are associated with elevated costs and entail a demand for highly specialized expertise contingent upon the specific technique and sample preparation prerequisites. Notably, these techniques can be classified into two principal categories: label-free methods, encompassing all electron microscopy (EM) techniques and AFM, and labelling methods, as typified by fluorescence microscopy (FM). The choice of method depends on the precise research objectives and constraints, making it necessary to weigh the advantages and limitations inherent in each approach for optimal utilization in a given scientific context.

The focus of this study is the evaluation of an innovative, label-free and variant-independent assay, called direct virus capture (DVC), designed specifically for the qualitative detection of intact SARS-CoV-2 virions through light scattering using a nano eye device virus detector (NED-VD). The proof of concept behind the DVC assay consists of the affinity interaction between biological virus targets, such as ACE2 receptors printed on a glass coverslip substrate, and the SARS-CoV-2 S glycoproteins located on the outer surface of virions. Consequently, any intact virions present in human swabs that bind to the glass coverslip can be detected by the NED-VD device. A detailed description of the NED-VD and its working principle have been provided in a previous publication [18], as well as the label-free detection of HIV pseudotyped with SARS-CoV-2 S proteins and whole SARS-CoV-2 virions, achieving a minimum limit of detection (LOD) of ~10^7 viral particle/ml.

Materials & methods

Specimen types

Between November 2021 and December 2022, a total of 152,284 nasopharyngeal swab specimens from different subjects were collected in 3 ml Copan Universal Transport Medium (UTM®) at the Microbiology and Virology

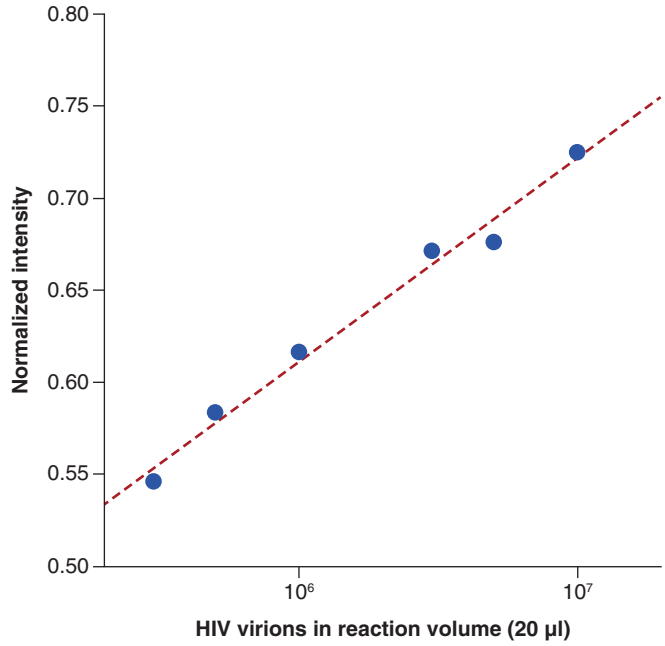

Figure 1. Pseudotyped HIV-1 calibration curve.

Department, Fondazione IRCCS Policlinico San Matteo, Pavia. Among these, 191 were included in this study and tested with NED-VD within 48 h from collection time, or after being stored at -80°C.

SARS-CoV-2 nucleic acid detection

All 191 human swabs specimens involved in the study were at first tested by RT-PCR assay to investigate the presence of SARS-CoV-2 genome, 170 of which were RT-PCR positive with Ct ranging from 14 and 25 (10^4-10^7 copies of RNA/ml) and 21 were RT-PCR negative. RNA extraction was conducted using the MGISP-960 automated workstation with the MGI Easy Magnetic Beads Virus DNA/RNA Extraction Kit (MGI Technologies, Shenzhen, China). The identification of SARS-CoV-2 RNA was performed using the SARS-CoV-2 variants ELITe MGB® kit (ELITechGroup, Puteaux, France; cat. no. RTS170ING), which targets the *ORF8* and *RDRP* genes. The reactions were carried out on the CFX96 Touch Real-time PCR detection system (BioRad, ON, Canada).

NED-VD

Nano eye device-virus detector (NED-VD) is an optical inverted microscope equipped with a 520 nm semi-telecentric laser line (Schäfter + Kirchhoff, model no. 5LTM-250-11 + 55CM-520-48-O08-A7.5-P-6). The optical coupling is patented [19], and developed specifically for the detection of spherical nanomaterials and nanoparticles down to 20 nm through light scattering. It is based on the propagation of an evanescent wave generated by the total internal reflection of the laser inside the substrate, which consists of a standard glass coverslip 0.17 mm thick. Since the evanescent wave has a penetration depth of the order of hundreds of nanometers, only the objects that are very close to the surface act as scattering centers. Digital images through six semi-apochromat objectives (UIS2, Olympus UPLFLN series) located on an automated revolver, ranging from 2× to 60× magnification. The images are then captured by a monochrome camera (IDS UI-3280CP-M-GL), with an exposure time of 200 ms. This specific geometry arrangement involving the laser beam, substrate, objectives and camera enables an efficient detection of nanomaterials and nanoparticles that are docked on the substrate [18]. In the DVC assay, when intact SARS-CoV-2 virions are immobilized on the substrate surface, they scatter the laser light that is collected by the microscope objectives.

Pseudotyped HIV-1 production

HIV-1 pseudotyped with the SARS-CoV-2 S protein was used to build the calibration curve (Figure 1). The virus was produced in HEK293T cells and for safety reasons, a HIV-1 molecular clone (*ENV*-defective pNL4-3), limited to single cycle replication was used. Later, pseudotyped HIV-1 was purified by overlay on a 25% sucrose cushion and ultracentrifugation (100,000 × *g*, 2 h). The number of particles of the purified virions preparation was assessed

by RT-assay (SG-PERT) while the concentration was evaluated by detection of the viral RNA by RT-PCR. More details are available in reference [18].

Bioconjugation of ACE2 proteins on the substrate

In this study, as substrate, we used glass coverslips (60 × 24 mm, 0.17 mm thickness functionalized with aldehydic groups (Schott–Nexterion). The circular active regions on the substrate were defined using the ACE2 bioreceptor (Signal-Chem), at the concentration of 0.5 μg/μl [18]. Bioreceptors were applied to the coverslip surface using drop casting through manual deposition, with a volume of 0.5 μl. The drops were dried at 25°C while shaking the coverslip at 1200 r.p.m. in a thermo-shaker (Bio-San PST-100HL). Following the drying process, a circular spot emerged, delineating the active area on the coverslip [18]. The size of the spot size was determined by the drop volume, resulting in a diameter within the range of 1–2 mm. As a consequence of the manual bioreceptor deposition method, its random orientation is expected, thus limiting the amount available for virus docking. Both quality and stability of ACE2 receptors were evaluated in the preliminary study to the one presented in this manuscript, using two types of viruses [18].

DVC assay

Glass coverslips were divided in 12 separated cylindrical wells of 6 mm in diameter using a silicone isolator gasket and a reaction chamber with springs (Grace Biolabs). To capture SARS-CoV-2 intact virions, the coverslip regions outside the spot were blocked to prevent non-specific adhesion (blocking step). After removal of the blocking solution the nasopharyngeal swab extract was dispensed and incubated (reaction step). During the incubation phase, the reaction chamber was sealed, thus preventing evaporation and cross-contamination between adjacent wells. Finally, the coverslips were washed with distilled water (dH_2O) to remove unbound or fragments of virions, or other nanomaterials potentially present in the starting sample, such as crystals or clusters of salts in the buffer solution, to avoid stray light or unwanted centers of light scattered.

As first step (blocking), 50 μl of blocking solution (phosphate buffer saline [PBS], 1% BSA, 0.05% V/V Tween-20) was dispensed in each well. Subsequently, the entire reaction chamber underwent incubation in a thermo-shaker (Bio-San PST-100HL) for 15 min at 25°C with 250 r.p.m. agitation. Each well was then subjected to two washes at room temperature, with 100 μl of washing buffer (PBS, 0.05% Tween-20) [18].

As second step (reaction), 40 μl of swab extract was added to each well, followed by incubation of the reaction chamber in the thermo-shaker for 30 min at 34°C with agitation at 1000 r.p.m. After the incubation time swab extract was removed from each well.

Finally, the reaction chamber was dismantled, the glass coverslip recovered and immersed for a wash in a 50 ml conical tube filled with dH_2O for 3 min at room temperature, then dried with air and inserted in a support before reading with NED-VD.

The DVC assay was entirely performed under a fume hood within a biosecurity level 2 environment. Reading by NED-VD was performed at room temperature, either on-site, just beside the hood, and remotely by exploiting the device's capabilities in IP sharing.

Intensity values spot calculation

In the DVC assay, the signal intensity within the spot area is related to the number of analytes captured, which are represented by intact SARS-CoV-2 virions.

In the typical digital workflow of the DVC assay, the slide is initially examined at 4× magnification to check for the possible presence of virions adhering on ACE2 spot. Eventually, if more detailed analysis is needed, higher magnification objectives (10–60×) are used.

For each spot, a signal intensity is evaluated using the formula $I_S/(I_S + I_B)$, where I_s is the mean intensity signal measured inside the spot, and I_B is the mean intensity value obtained in the area surrounding the spot (background). Both I_S and I_B are automatically measured by the proprietary software developed to control the device. If the resulting value is above a defined threshold the sample is considered as positive.

A representative example of a spot that is positive and one that is negative in the DVC assay is shown in Figure 2A and B, respectively.

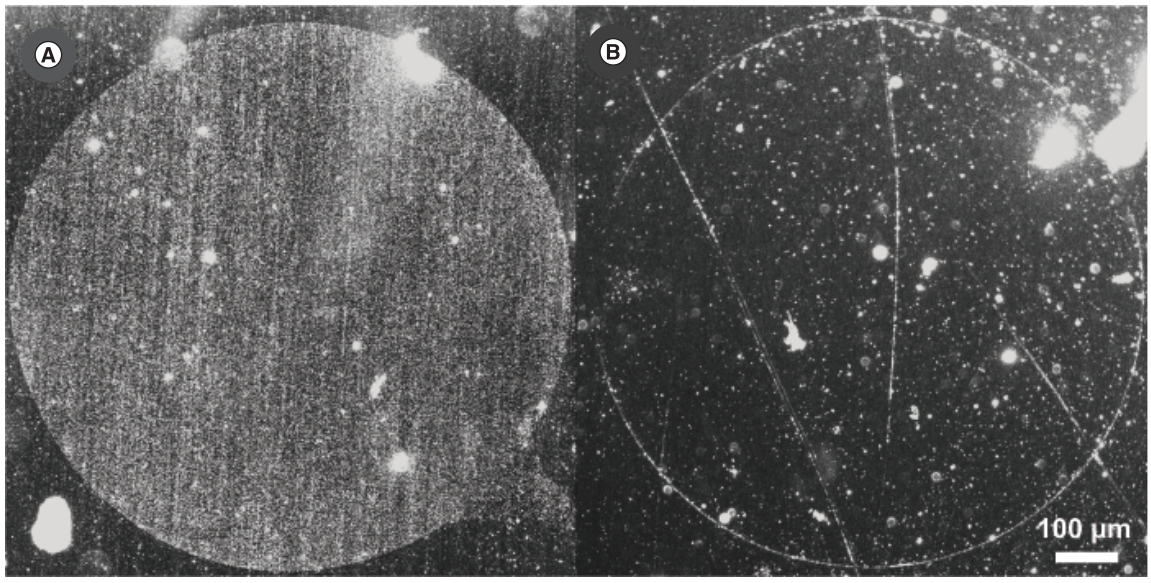

Figure 2. Example of images captured with nano eye device-virus detector for positive and negative spot in the direct virus capture assay.

Table 1. Confusion matrix for the direct virus capture assay.

		Actual values	
		Positive	Negative
Predicted values	Positive	TP	FP
		69	2
	Negative	FN	TN
		101	19

The confusion matrix below summarizes the performance of DVC assay showing data relative to sensitivity (TP / TP + FN), specificity (TN / FP + TN) and accuracy (TP + TN / TP + FN + FP + TN). Sensitivity = 40.5%; specificity = 90.4% and accuracy = 46%.
FN: False negative; FP: False positive; TN: True negative; TP: True positive.

Evaluation of DVC assay

To establish the cut-off values, sensitivity, specificity and accuracy of the assay, a comparative analysis between the data obtained from RT-PCR tests and the results of the DVC was conducted. We focused on the specimens that tested negative by RT-PCR to initially define the DVC method's cut-off point: based on the methods described in the previous paragraph a value of 0.50 ± 0.02 was determined. Below this cut-off, the tests were considered negative, and above it, the test was considered positive. Subsequently, the same specimens that tested negative by RT-PCR were used to determine the specificity of the DVC assay. In contrast, the human swabs that tested positive by RT-PCR, with corresponding Ct values, were used to evaluate the sensitivity of DVC assay. Finally, we utilized all the information gathered from human swabs tested both positive and negative by RT-PCR to assess the overall accuracy of the DVC assay. Cross reactivity of ACE2 toward other targets was beyond the scope of this study.

Results

The DVC assay showed 40.5% concordance with positive RT-PCR results and 90.4% concordance with negative RT-PCR results (Table 1). The corresponding DVC assay results for the 170 RT-PCR positive swabs, along with their respective Ct values, are reported in Table 2. To determine the minimum detectable concentration of whole virions, we used serial dilutions of HIV-1 virions pseudotyped with the SARS-CoV-2 S protein (Figure 1). As expected, the DVC assay exhibited higher agreement with PCR results up to Ct values of 21 (corresponding approximately to 10^7 copies/ml), after which the agreement gradually decreased (Figure 3).

Surprisingly, even at low Ct, we never observed a 100% concordance in results. This finding is not related to the assay sensitivity but rather to the detection of viral particles as compared with genetic footprints, as discussed in

Table 2. Panel of 170 swabs RT-PCR positive with related Ct values, and corresponding DVC assay results with positive/negative outcome.

RT-PCR positive	DVC	
Swabs, n (Ct values)	Positive (n)	Negative (n)
5 (14)	3	2
9 (16)	4	5
13 (17)	8	5
27 (18)	14	13
32 (19)	16	16
31 (20)	12	19
13 (21)	5	8
20 (22)	3	17
9 (23)	2	7
7 (24)	1	6
4 (25)	1	3

DVC: Direct virus capture.

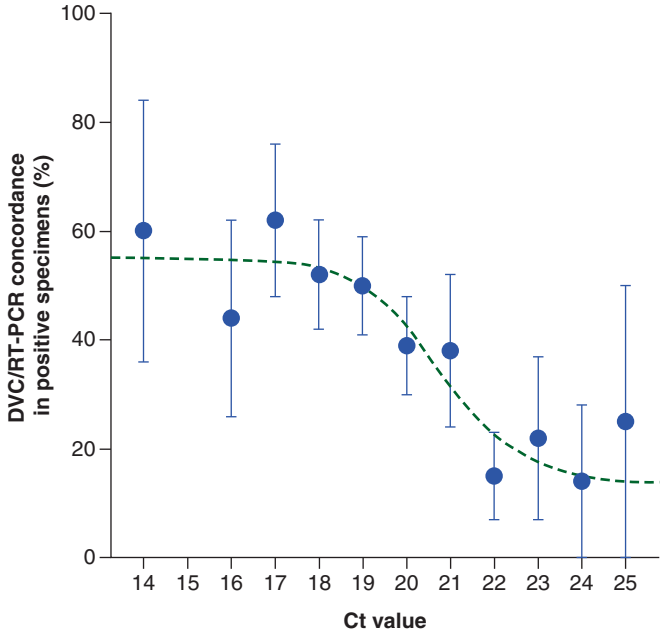

Figure 3. Direct virus capture. PCR assay agreement in positive specimens.
DVC: Direct virus capture.

the next section. We present in Figure 4 the images and the corresponding normalized signal of the most intense spot at each Ct, including also one negative.

There were no qualitative differences in results between fresh and frozen swabs, and the repeatability of DVC test was experimentally performed two-times for each patient to confirm the qualitative reproducibility of the assay. We did not intentionally test interfering pathogens, that can be accidentally contained in collected swabs.

Discussion

Here, we present a label-free biosensor able to detect through light scattering intact SARS-CoV-2 virions, independently from the variant, in human clinical samples showing a sensitivity of 40.5%, specificity of 90.4% and accuracy of 46% if compared with gold standard RT-PCR.

The merit achieved of the DVC assay can be explained by three main reasons. First, it is well known that the SARS-CoV-2 virus specifically attaches to the host cell ACE2 receptor by the RBD fragment present in the S1 subunit of its S protein which cover the outer virion's membrane [5]. Due to this, the DVC assay takes advantage of the biological high affinity between RBD S protein and ACE2 host cell receptor to detect whole SARS-CoV-2

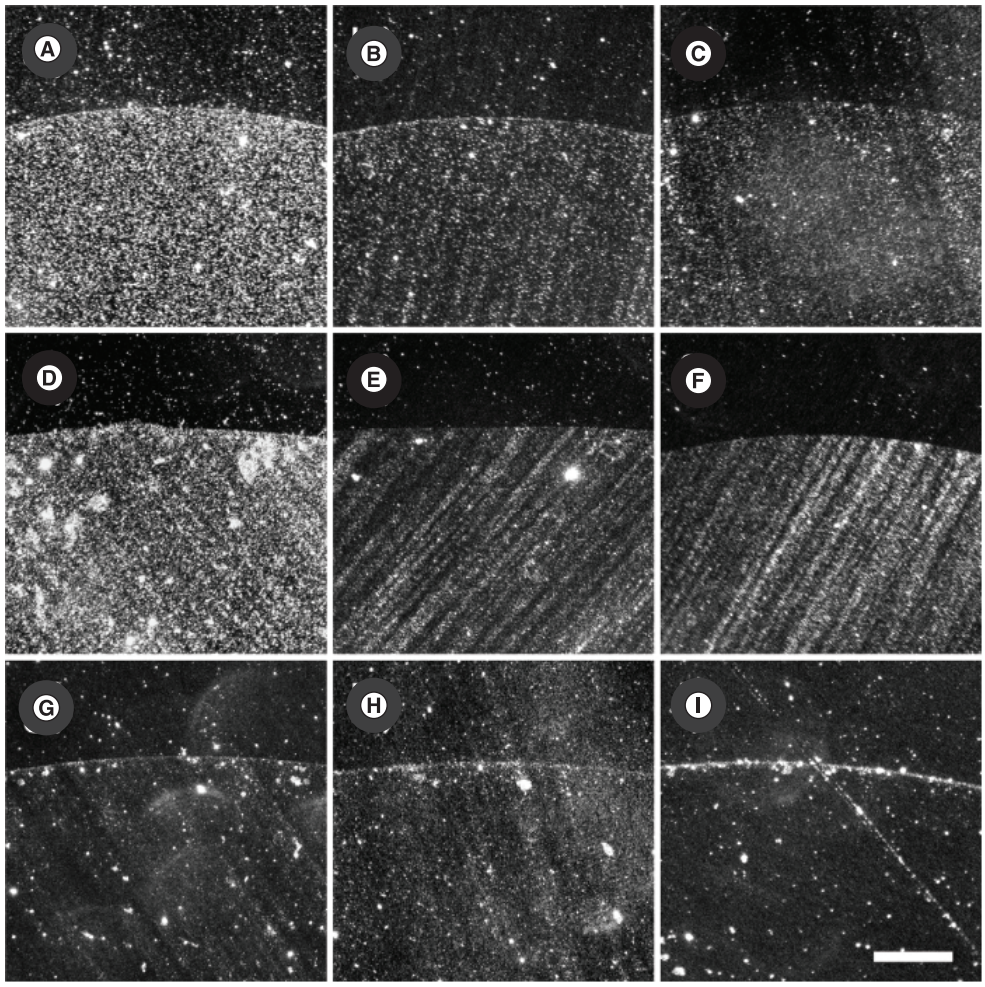

Figure 4. Direct virus capture. Assay images of the most intense spot at each Ct and one negative.

virions. Second, we can detect only the fully formed virions that, at the end of their life cycle, are transferred by exocytosis in the extracellular environment [20]. We don't use any pre-analytical step to lyse cells in order to detect viral material inside the host cell. Third, the optical laser coupling embedded in the NED-VD device is able to detect spherical nanoparticles >20 nm through light scattering [18,19], with no signal contribution coming from smaller non-spherical objects, such as organic proteins; in fact, SARS-CoV-2 virions have a spherical shape with a diameter of about 130 nm [4,5]. Instead, ACE2, free S proteins, not fully formed or broken SARS-CoV-2 virions, cannot act as specific signal emitters because proteins are too small and broken virions are not spherical so they don't scatter enough light to be detected by NED-VD optical system. Since the target of DVC assay is only the SARS-CoV-2 fully formed virion, it is clear that the concordance with RT-PCR will never achieve 100%, even at Ct <21, because the detection of viral nucleic acids does not necessarily imply the presence of intact virions in clinical specimens [21–26]. In fact, the Ct value is just an indirect marker of the potential infectivity, because does not measure the number of viable virions present within a sample [27]. It is actually considered only an estimation of the viral load by measuring it in RNA copies/ml [7], as it is known that only intact SARS-CoV-2 virions are capable of entering host cells and replicating, making the patient infectious. The detection of intact virions is a critical factor in accurately identifying infectious individuals who may be transmitting the virus [28,29].

Considering the well-known kinetics of RNA viral load and infectious virus of SARS-CoV-2 [24,25], the first week after infection is the time window useful to perform DVC test given the higher quantity of virions fully formed that can be captured, providing a positive outcome. Whereas, after 2–3 weeks after infection, only virus RNA will be present, thus resulting positive at PCR and negative at DVC. After several weeks post infection, traces of RNA were found in respiratory samples, as opposed to the short lifespan of virions and these evidences find clear confirmation

in scientific works [25,29]. Furthermore, it is known that RNA counting by PCR can markedly overestimate the number of virions and can also be present within the sample in three main forms [8]. Even if the fraction of intact virions is not necessarily related to the amount of genetic material, when considering a large population of samples, it was expected that on average such correlation naturally occurs. In fact, even during the experimental session of DVC, a large variability of spot intensity in samples with the same Ct values was noticed, as summarized in Table 2. This is also the reason why we observe a decrease in the concordance between DVC and RT-PCR at Ct >21 (Figure 3). As further confirmation, the spot intensity values do not follow the Ct values trend, as shown in Figure 4.

Considering the detection method and the target of RT-PCR, it would be probably more useful to compare DVC with other techniques, like antigenic tests, which share more similarities than a molecular assay. Even in this case, when considering an antigen/antibodies test (e.g., LFA or ELISA), a discrepancy in sensitivity due to the nature of the researched analyte would be still expected. Indeed, the best qualitative antigen/antibodies kits are generally sensitive up to Ct values of ca. 25–30 [30]. If compared with DVC, the higher sensitivity of the antigenic test is merely due by the fact that one single virion can contain up to about 100 S and 1000 N proteins [31], so a consistent gap of 7–10 levels of Ct in sensitivity was expected. Moreover, like for PCR test, it is important to underline that the target of an antigenic test is represented by traces of the presence of the virus, not by the virus itself. The most common COVID-19 diagnostic strategies, such as PCR, LFA or ELISA assays need fluorescent dye, colorimetric and chemiluminescence signal, respectively, in their detection approach. Instead, one of the most advantage of DVC method, considering time, cost, and easy to perform, is the detection in light scattering of whole SARS-CoV-2 virions in a label-free mode based on the signal intensity of the analytes captured within the ACE2 bioactive area [18,19].

Beside these diagnostic tests, currently other techniques and devices are used in research to detect the presence of SARS-CoV-2 in a label-free mode: among other laboratory techniques, the viral culture provides direct information about the effective presence, replication and infectivity of the live virus within the samples [4,6] but several days are necessary for the results. About devices able to directly detect and image virions, TEM offer the best resolution, but requires preparation of the sample by placing each one under vacuum, whereas AFM can take up to 15–20 min for the acquisition of a single image of the sensing spot. Despite that these latter are label free methods, they require very expensive devices or long investigation time so they cannot be used in a clinical daily routine. On the other side NED-VD needs only 200 milliseconds of exposure time to capture the image of a spot. Finally, the most important advantages of DVC are its unicity as test searching for virions in a swab, with no use of labels so lower cost per test, lower time for optical detection compared with PCR, and less stringent competence requirements for the user. In general, future improvements in sensitivity, specificity, and accuracy will therefore be necessary for the application of the DVC method for clinical purpose.

Conclusion

The new virological biosensor proposed is a proof of concept as preliminary screening test to evaluate the presence of whole virions within the human swabs. It is based on the high biological affinity that occurs between the ACE2 bioreceptor and the S proteins located on the outer membrane of SARS-CoV-2 virions. At the current development stage results given by DVC test are not yet sufficient for diagnostic purposes. Besides all the possible further steps required to increase sensitivity, the technique proposed here has several possible advantages. DVC test could possibly be used as a pre-screening test to evaluate the presence of active virions and reducing the number of PCR tests, also considering that this test is cheaper (about 1/10 the cost of a PCR), faster (60 min), and not dependent on the specific variant of the virus. In this perspective, even a 40.5% sensitivity could be a great advantage in terms of saving time and cost. One of the future developments is the miniaturization of the entire assay with the consequent reduction of required volumes, by further matching the spot area to the size of the bottom well. In addition, antibody-oriented immobilization by using aptamers may improve the sensitivity of DVC assay by reducing steric hindrances, reducing non-specific binding, increasing the receptivity of the biosensor, and enhancing the signal-to-noise ratio of the assay [32]. Overall, we can assume that the detection of virions through DVC assay can be extended to all spherical viruses having a sufficient detectable signal in light scattering by NED-VD, specifically those with a minimum size of at least 40 nm.

Summary points

- The direct virus capture (DVC) assay is a new, fast and variant-independent virological method capable of detecting intact SARS-CoV-2 virions within human swab specimens in a label free manner, solely through light scattering, using the nano eye device virus detector (NED-VD) device.
- The performance of the DVC test, when compared with the gold standard RT-PCR, exhibits strong concordance up to Ct value of 21, but this concordance diminishes as Ct values increase.
- Comparing the DVC assay with viral culture could offer valuable insights into the infectivity of virions detected by the NED-VD.
- Enhancing the sensitivity and automation of the DVC test could enable its integration into routine clinical workflow.
- The working principle of this new assay can be extended to all spherical microorganisms with a diameter at least 40 nm.

Author contributions

A Pennesi: writing - original draft, data curation, visualization. G Ferrari: validation, investigation. F Giardina: validation, resources, investigation. S Piselli: investigation. R Lo Savio: formal analysis, writing – review and editing. A Carloni: conceptualization, methodology, writing – review and editing, project administration. S Paolucci: supervision, project administration. F Baldanti: supervision, project administration.

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Competing interests disclosure

The assay tested in this study is currently in development at NTP Nano Tech Projects. The authors have no other competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript apart from those disclosed.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

This study was approved by the Ethics Committee of Fondazione IRCCS Policlinico San Matteo, Pavia, Italy (#43923/2021).

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