Application of flow-virometry for large-scale screening of COVID 19 cases

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Abstract:

The coronavirus disease caused by SARS-CoV-2 (known as COVID 19) is highly contagious and has spread rapidly over 200 countries over last three months. WHO suggested urgent escalation in testing, isolation and contact tracing, as the "backbone" of managing the pandemic. Globally, the detection of SARS-CoV-2 in patients are done by RT-PCR and blood antibody-based testing. In addition to the existing processes, flow-cytometry could be used as a high-throughput and efficient diagnostic method for detection of COVID 19. The suspected COVID 19 samples can be analyzed using 'Indirect flow cytometry', with the specific primary antibody and fluorescent tagged secondary antibodies. The fluorescence signal can distinguish the infected v/s non-infected samples. In the present article, we have summarized the applications of Flow virometry to study various viruses and have proposed possible application in the detection of COVID 19.

Introduction:

Flow cytometry is a laser-based technology which allows extremely rapid measurement of single cells. Fluorescence-Activated Cell Sorting (FACS) have been method of choice since the 1970s to analyze and purify cells of interest. It can detect and discriminate cells by light scattering, auto fluorescence and or fluorescent properties of a cell or a particle. In the latter case, fluorescence is achieved with antibodies and fluorescent secondary reagents directed at the cell surface and/or internal constituents in permeabilized cells. Alternatively, the use of cells encoding genetically modified proteins that are fluorescently tagged (eg. Green fluorescent protein - GFP) has also been proven to monitor cells using flow cytometry (Lippé, 2018). The analysis of viruses by flow cytometry was termed as "flow virometry" (Arakelyan *et al.*, 2013).

The recent coronavirus pandemic COVID 2019 has spread rapidly across the world and almost every country is affected. This virus is highly contagious and has unique clinical characteristics with key pathological mechanism which remains unclear. Until a successful treatment strategy evolve the key of managing this pandemic is greatly dependent on quick and faster detection of infected individuals followed by isolation of patients from healthy population. Presently the detection of the COVID 19 is done by qRT-PCR using unique set of PCR primers. The test detects the viral RNA in the samples. There are several kits in the market for the detection of SARS-CoV-2 using this method. Detection of SARS-CoV-2 antibodies in human blood is another method being used for developing rapid detection kits. Few weeks before an RT-LAMP kit has been launched which uses isothermal amplification of viral nucleic acid.

Although RT-PCR based detection and blood antibody detection of SARS-CoV-2 virus are being practiced successfully but to cover the larger population we need a high-throughput, faster, specific and sensitive test for the coronavirus detection. Here we propose, the use of Flow cytometry as a possible powerful tool to diagnose the SARS-CoV-2.

Materials and Methods:

Flow Virometry:

The use of flow cytometry to characterize viruses was pioneered decades ago. Flow cytometer were used to detect 70X200 nm long T2 phages fixed with glutaraldehyde- or formaldehyde (Hercher *et al.*, 1979). Flow virometry has now been used to characterize an expanding array of viruses, including lambda phage, herpes simplex virus 1 (HSV-1), mouse hepatitis virus (MHV), human immunodeficiency virus (HIV), Nipah virus, Junin virus, vaccinia virus, dengue virus, human cytomegalovirus (HCMV) and giant viruses. This requires careful sample preparation, fixing, labeling of the viral particles, and heating to promote the penetrance of the dye. Flow virometry is indeed becoming a powerful tool to study, sort and characterize Viruses (Lippé, 2018).

Virus studies using Flow cytometry:

Virus particles or virion could be detected using flow cytometry either based on the size or by fluorescent labelling. Table 1 represents some examples where flow cytometry was used for detection of virus particles of various shapes and sizes. Labelling of genetic materials (DNA/RNA) and viral capsid using fluorescent lipophilic markers, antibodies, magnetic nano-particles (MNP) tagged with fluorescent immunoglobulins for sorting, and detection of virus particles are summarized as below.

Size and reduced wide-angle (Forward Light Scatter) FSC:

Size of the virus particles (virion) ranges from smallest non-enveloped 17-nm circovirus (Tischer *et al.*, 1974) to the larger enveloped vaccinia viruses 350 nm (Dubochet *et al.*, 1994), to the giant viruses which is 1 µm in size (La Seola *et al.*, 2003; Philippe *et al.*, 2013).

In standard flow cytometers, detectors of forward light scatter (FSC) depicts the size of a cell or a particle passing through the flow channel. The small sized viruses when analyzed on flow cytometer falls in the range corresponding to optical, electrical, and filtered sheath buffer background noise. FSC detectors

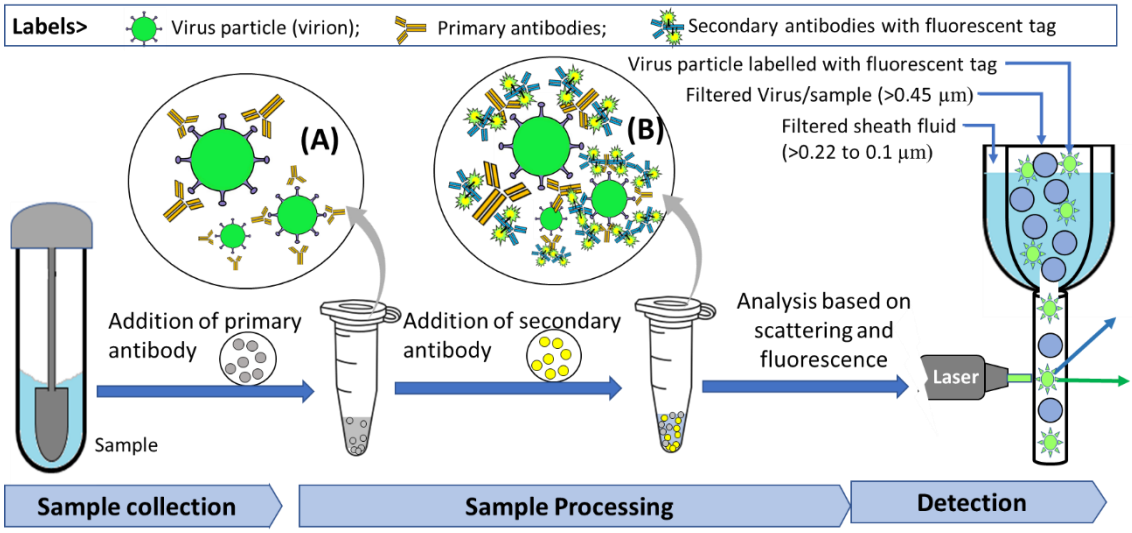

Figure 1: A process flow diagram showing detection of SARS-CoV-2 virus using flow-cytometer. Samples with virus transport media mixed with primary and secondary antibodies. The Secondary antibodies are labelled with fluorescent tag and could be analyzed using flow cytometry. (A) Binding of surface protein of virus with primary antibodies; (B) Binding of primary antibodies with fluorescent tagged secondary antibodies.

typically monitor light in the 0.5° to 15° range, where most of the background signal is found. Latter, the concept of "reduced wide-angle FSC" detection, which blocks light in the 0° to 15° range to reduce noise and monitor light at angles between 15° and 70° was demonstrated (Lippé, 2018). This has greatly improved the signal-to-noise ratios. To further reduce the signals from impurities, filtering the sheath buffer with a 0.1 µm filter, instead of the standard 0.22 µm filter, were suggested for the study of smaller sized viruses. Similarly, filtering samples themselves with a 0.45-µm cutoff, when possible, helps to limit the aggregates and artifacts (Loret *et al.*, 2012; El Bilali *et al.*, 2017*)*.

Labelling Genetic content:

Another successful approach to detect viruses using flow cytometry is to label the genetic content of viruses. The viral nucleic acid is buried inside a protein shell (capsid), which is itself covered by one or several distinct proteins (called the matrix or tegument layer depending on the virus) and, for enveloped viruses, a lipid bilayer. Due to the capsid not all the nucleic acid dyes have been able to stain the Virus DNA/RNA. The examples of nucleic acid dyes used for labelling viral DNA/ RNA includes SyBR green-I, YOYO-1, TOTO-1, and PicoGreen (Brussaard *et al.*, 2000; Gaudin *et al.*, 2015; Marie *et al.*, 1996; Marie *et al.*, 1997*)*. However, report suggests that SyBR green-I does not always efficiently label viral particles unless they are first heated to up to 80 to 90°C (Marie *et al.*, 1999*;* Brussaard *et al.*, 2004). After screening a number of commercial dyes, the membrane-permeable Syto 13 (green fluorescence) or Syto 61 (red fluorescnce) works

best for labeling herpes simplex virus 1 (HSV-1). Both these dyes resulted good signals, very low background noise, and excellent sample penetrance (Loret *et al.*, 2012; El Bilali *et al.*, 2017). Most importantly, these Syto dyes are not detrimental to the infectivity of the virus at the concentration (1 µM) used.

Labelling with fluorescent lipophilic markers in the case of enveloped viruses:

The enveloped viruses can also be labelled using lipid dyes for flow cytometry analysis. This approach was successfully employed for HIV, vaccinia virus, and dengue virus using DiD, DiO, and DiI (Arakelyan *et al.*, 2013; Tang *et al.*, 2016; Zicaris *et al.*, 2016). PKH67, a green fluorescent molecule with an aliphatic tail that targets lipid bilayers, is one of the most commonly used dye for labeling of the enveloped (animal) viruses (Van der Vlist *et al.*, 2012; Nolte-'t Hoen *et al.*, 2013). This molecule could potentially be used for screening of SARS-CoV-2 as this virus has lipid bilayer.

Labelling with antibodies coupled to fluorescent moiety:

Labelling virus particles with primary antibodies (IgG/ IgM with a fluorescent tag) specific to capsid proteins is another approach which could be used successfully for screening. In case fluorescent tagging of primary antibody is not possible/available a fluorochrome tagged secondary antibodies which target primary antibodies (IgG/ IgM) could be used. In some cases, these antibodies have also been pre-bound to 15-nm nanobeads to improve the detection of the virus by light scattering (Arakelyan *et al.*, 2013).

Labelling with MNPs tagged with fluorescent immunoglobulins

Magnetic Nanoparticles (MNPs) have been used to perform the qualitative and quantitative measurements in virometry analysis. The technology is based on binding of the virus molecules to the magnetic beads. The magnetic beads are incubated with a virion specific

antibodies. The 'MNP-antibody' complex is then mixed and incubated with the virions for immobilization. The immobilized 'MNP-antibody-virion' complex is then incubated with secondary antibodies with fluorescent tags. Later magnetic bound virion complex is separated with magnetic column and analyzed in a FACS system. The use of MNPs has been reported in the study of HLA DR/LFA1 heterogeneity of HIV 1 virus (Arakelyan *et al.*, 2013).

Apart from enumeration of viruses, flow cytometry is being used to detect infection via blood lymphocyte subpopulation distribution studies. Viral infections result a range of immune

responses in the body and peripheral blood mononuclear cells (PBMC) are studied to score the level of infection. Flow cytometry could be used to analyze PBMCs, and fluorescence-activated cell sorting (FACS) is employed to sort each sub-population of cells. Similar study was done for biochemical indices and lymphocyte subpopulation distribution in the samples of COVID 19 infected patients, and compared the data with those where pneumonia was not caused by COVID 19 (Yishan *et al.*, 2020).

To better understand and facilitate discovery in the immune response to COVID 19, comprehensive research tools are made available. It includes solutions for immunophenotypic, transcriptional and functional analysis of immune cells which covers up the major areas like viral immune response, cytokine analysis, vaccine research, biomarkers and therapeutics. (BD biosciences). https://www.bdbiosciences.com/en-in/applications/research-applications/covid-19

With all the above understanding, we propose the detection of SARS-CoV-2 in the samples using antibodies coupled to fluorescent moiety.

Table 1: Detection of viruses using flow-virometry

Virus	Size (nm)	Detection process	Application	Reference
HIV-1 Virions	120-150	Label enveloped viruses- Lipid dyes (DiO, DiD)	HLA DR/LFA1 heterogeneity	Arakelyan *et al.*, 2013
HSV-1	125	Nucleic acid stain-Syto 13	Nuclear capsid sorting	Arakelyan *et al.*, 2013
HSV-1	250	GFP tagging to diverse virion Components	Tegument variability; viral fitness	Loret *et al.*, 2012
HIV-1 Virions	120-150	Magnetic nanoparticles (MNPs)	HLA DR/LFA1 heterogeneity Envelope conformation	El Bilali *et al.*, 2017
Dengue virus	40–60	Fluorescent lipidic dye-DiI, Fluorescently labeled 2H2anti-prM antibodies, Magnetic nanoparticles (MNPs)	Virion maturation	Zicari *et al.*, 2016; Arakelyan *et al.*, 2017
T4 (Bacteriophages)	70 X 200	Nucleic acid stain-SYBR Green I	Single-virus genomics	Allen *et al.*, 2011

Results and Discussion:

Process and method of COVID 19 detection:

In the current report, we are highlighting a flow virometry based COVID 19 detection strategies. Report suggest that SARS-CoV-2 belong to the family *Coronaviridae*, consisting of positive-sense single-stranded RNA of approximately 29–30 kb. The virus particle size ranged from 70–90 nm (Jeong-Min *et al.*, 2020). Extensive studies on dengue virion (40-60 nm) was done using combination of fluorescently labeled antibodies and magnetic nanoparticles (MNPs) (Zicari *et al.*, 2016). Here we propose the approach of labeling the surface of the viral particles with antigen specific primary antibodies and secondary antibody conjugated to a fluorescent dye (eg: FITC, PE, Cy5®, etc.) to detect the SARS-CoV-2 virus.

(a) Collect the sample from the oral/nasal cavity of the suspected patients and transfer to the testing point in tubes containing viral transport media. Re-suspend the sample in the ice-cold phosphate buffer saline (PBS) solution and 1% sodium azide (report suggests, ice cold PBS and 1% sodium azide helps to prevent the modulation and internalization of surface antigens which can produce a loss of fluorescence intensity).

(b) Add 0.1-10 µg/ml of the primary antibody. Dilutions, if necessary, should be made in 3% BSA/PBS and incubate for at least 30 min at room temperature or at 4°C in the dark. Wash the cells 3 times by centrifugation at 400 g for 5 min and re-suspend them in ice cold PBS.

(c) Dilute the fluorochrome-labeled secondary antibody in 3% BSA/PBS at the optimal dilution (according to the manufacturer's instructions) and then re-suspend the cells in this solution. Incubate in dark for at least 20-30 min at room temperature or 4°C. Wash the cells 3 times by centrifugation at 400 g for 5 min and re-suspend them in ice cold PBS, 3% BSA, 1% sodium azide.

(d) Analyze the cells on the flow cytometer as soon as possible. {Note: One need to adjust the conditions of the centrifugation (force and time) with respect to the virus}.

(e) For Virus studies, filtering the sheath fluid with 0.1 µm instead of 0.22 µm and filtering samples themselves with a 0.45 µm cutoff, limits aggregates and artifacts. Viruses being small in size, proper thresholds needs to be set for Forward and Side scatter. For example, for T4/lambda particle (70 X 200nm) FSC PMT was set at 1000 and SSC at 200 to maximize signal-to-noise ratios.

(f) Controls: Prior to sample analysis, blank: i.e. filtered PBS needs to be analyzed for background event recognition. The analysis needs to be done at low flow rate and Readings captured on bi-exponential plots for fluorescence signals and linear scale for FSC and SCC.

Flow cytometry could detect positive-polarity RNA dengue virus (DENV) after 24 h post infection in Vero 76 (African Green monkey kidney) cells. The detection was made possible using fluorescein isothiocyanate (FITC)-labeled 4G2 monoclonal antibody (Lambeth *et al.*, 2005). Similarly, we expect the early detection of SAR COV-2 in suspected patients using the proposed concept.

Conclusion:

We hypothesize the use of flow virometry to detect the SARS-CoV-2 in the infected samples. The approach of indirect immunofluorescence using specific primary antibody and the fluorescent tagged secondary antibody (specific binding to primary antibodies) would give faster and sensitive detection for coronavirus. Outline of process flow is depicted in Fig. 1. The process of labelling the antibody is expected to take 1.5 to 2 hours and after the labelling the sample analysis time on flow cytometer is about 30 seconds. High-throughput sample processing and analysis in a 96 well plate could be done in 2 – 3 hours with a robotic liquid handler. Therefore, the method described here will enable high-throughput (1500 – 1800 samples a day) and robot assisted screening COVID 19 cases. However, further optimization of the process for pooled sample analysis could help to reach 10 – 20 times more samples per day.

Overall process could be automated with a liquid handler equipped with a robotic arm to minimize human interaction and achieve throughput. Establishment of this process will also help in reducing dependencies on qRT-PCR machines and reagents. In countries like India where populations density is very high, quick and large-scale sample analysis is highly required to curb the spread of the virus. The method described here will also be useful for community studies in metropolitan cities, hospital staffs, and the people who are associated with emergency services in this unprecedented time across the globe.

Conflict of interest: The authors declared that no conflict of interest exists regarding this publication.

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Abbreviations:
FACS (Fluorescence Activated Cell Sorting); DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt); DiO (3,3'-Dioctadecyloxacarbocyanine Perchlorate), and DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ('DiI'; DiIC18(3)));YOYO™-1 Iodide {1,1'-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis[4-[(3-methylbenzo-1,3-oxazol-2-yl)methylidene]-l,4-dihydroquinolinium] tetraiodide}, PKH67 (Green Fluorescent Cell Linker), IgG: Immunoglobulin G, IgM: Immunoglobulin M; HIV, Human Immunodeficiency Virus; SARS-CoV-2, Severe Acute Respiratory Syndrome Corona Virus 2

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