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A Flow Virometry Process for Detection of SARS-Cov-2 Proposed for Large-Scale Screening of COVID-19 Cases

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Abstract- The coronavirus disease, COVID 19, caused by SARS-CoV-2 has spread rapidly over 210 countries and declared as pandemic by World Health Organization (WHO). WHO also emphasized on scaling-up testing capacity, followed by isolation of infected individuals, 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. However, immediate expansion of the scale of screening is very important to cover major fraction of 1.3 billion population of India. We, therefore, propose a flow-cytometry based high-throughput screening systems for testing of COVID 19 cases. The swab-samples in viral transport media could be analyzed using 'indirect flow cytometry', where virus particle binds to specific primary antibody and the resulted virus-antibody complex then binds to a fluorescent tagged secondary antibodies. The fluorescence signal can distinguish on a flow-channel to identify viral load in a test sample. In the present article, we have summarized a flow-virometry process for detection of various viruses and have proposed possible application of the process in screening of COVID 19.

Keywords- COVID-19, Severe acute respiratory syndrome (SARS), SAR-CoV-2, flow Cytometry, flow virometry

I. INTRODUCTION

Flow cytometry is a laser-based cell biology technique to analyze, count and sort cells of interest from a mixed population. It has gained the popularity as it is rapid and quantitative method for analysis and purification of cells in suspension. Since 1970s Fluorescence-Activated Cell Sorting (FACS) have been method of choice successfully used to analyze and purify plant and animal cells. It can detect and discriminate cells by light scattering, auto fluorescence and or fluorescent properties of a cell or a particle. The fluorescence is obtained with specific antibodies (against cell surface and/or internal constituents in permeabilized cells) and fluorescent secondary reagents. Also, the technique is used in monitoring cells encoding genetically modified proteins that are fluorescently tagged (eg. Green fluorescent protein - GFP) [1]. In a variety of biomedical, clinical, and therapeutic research FACS has been immensely used for generating reliable data. Flow cytometry is also a powerful technique for correlating multiple characteristics on single cells. The information obtained is both qualitative and quantitative which has widened its applications from research to clinical studies. Fluorescent dyes are used for various flowcytometry analysis as DNA cell cycle analysis, indirect/direct immunophenotyping, apoptosis study and transformation using fluorescent molecular markers. The analysis of viruses by flow cytometry was termed as "flow virometry" [2,3].

The recent coronavirus pandemic COVID 2019 is induced by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It belongs to the Sarbecovirus subgenus (genus Betacoronavirus, family Coronaviridae). This virus has unique clinical characteristics, highly contagious with unclear pathological mechanisms. The virus can cause a life-threatening respiratory illness in humans especially to the people at late 50s and above or/and with comorbidity (diabetes, kidney diseases, heart diseases etc.). Early symptoms of SARS-CoV-2 infection is very similar to that of mild to moderate flu which makes it extremely difficult to classify and shortlisting infected individuals. In addition, inefficient contact tracing of infected individuals is becoming increasingly hard.

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, and the first step of the process is making cDNA from viral RNA using reverse transcriptase enzyme. Detection of SARS-CoV-2 antibodies in human blood is another method being used for development of rapid detection kits using lateral flow immunochemistry. Reverse Transcriptase – LAMP (loop-mediated isothermal amplification) is another potential detection system for SARS-CoV-2 in which uses isothermal amplification of

viral nucleic acid using especially designed oligo-nucleotide primers. Several PCR (polymerase chain reaction) based high-throughput diagnostics kit for detection of SARS-CoV-2 is also under development [4].

Although, blood antibody detection and qRT-PCR assay for detection of SARS-CoV-2 virus are being practiced successfully but to cover the larger population we need an acurate, high-throughput and faster sample testing for the coronavirus detection. India is among the top ten countries in respect to the number of COVID 19 cases and over last two months the rate of infection is growing exponentially. At present, India is testing little over three thousand people per million of population, which is clearly insufficient, and therefore a large-scale and robust screening process need to be developed.

Recently, A human monoclonal antibody blocking SARS-CoV-2 infection has been reported. These antibodies binds to a conserve receptor-binding domain (RBD) on the spike of the virion, and thus results specificity to SARS-CoV-2 [5]. Such monoclonal antibodies against SARS-CoV-2 shall be useful for development of antigen detection tests and serological assays. Döhla *et al*. (2020) reported that 88.9% specificity in qRT-PCR whereas 36.4% specificity in antibody based rapid detection kit for diagnosis of COVID 19 cases [5]. Here, we propose an approach for diagnosis of SARS-CoV-2 in screening of test samples for COVID 19 samples using flow cytometry. In this approach, we have employed the process of indirect immunofluorescence where the virus particles were first bind to specific primary antibodies and then labelling the complex by fluorescent secondary antibodies for the detection in a flow-cell.

II. RELATED WORK

Flow Virometry:
Flow cytometer were used to detect 70X200 nm long T2 phages fixed with glutaraldehyde- or formaldehyde [5]. Characterization of viruses using flow cytometry were pioneered decades ago. Advanced Flow virometry has now been used to characterize several viruses such as lambda phage, human immunodeficiency virus (HIV), herpes simplex virus 1 (HSV-1), mouse hepatitis virus (MHV), vaccinia virus, dengue virus, Junin virus, human cytomegalovirus (HCMV), Nipah virus, and giant viruses. Fixing, labeling of the viral particles, careful sample preparation, and optimized heating to promote the penetrance of the dye in the virion is the most important step. For characterization, sorting, and study of viruses flow virometry is emerging as a powerful tool for future [1].

Flow cytometry to study viruses:
Virus particles could be detected in flow cytometer either based on fluoresce or size of the particle. There are ample examples where virus particles of various shapes and sizes were sorted or detected using advance flow cytometry methods (Table 1). Labeling of viral capsid using fluorescent lipophilic dye, labeling genetic materials (DNA/RNA) using nucleic acid binding dye, fluorescent immunoglobulin tagged MNPs (magnetic nano-particles) and few widely used methods for detection of virus particles are described below.

Forward Light Scatter (FSC) and reduced wide-angle for size:
Virions (virus particles) shows a great variation in sizes ranging from 15 nm for non-enveloped circovirus [7] to 350 nm for larger enveloped vaccinia viruses [8], to 1 µm giant viruses [9,10]. 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. Typically, FSC detectors monitor light in the 0.5 - 15° range and most of the background signal are found in this range. 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 [1]. This has greatly improved the signal-to-noise ratios. Impurities from signal could further be reduced by filtering the sheath buffer with a 0.1 µm filter, instead of 0.22 µm filter which is routinely used, is highly recommended for the study of virions of smaller sized. In addition, filtering test samples with a 0.45 µm pore, when possible will be very useful to reduce aggregates and artifacts during analysis [11,12].

Labelling Genetic content:
Labelling the genetic content of viruses is another most efficient approach to detect virions in sample using flow cytometry. The viral nucleic acid is enclosed inside the protein capsid, and the capsid often covered with one or several distinct layers of proteins which is known as matrix or tegument layer. Almost all enveloped viruses contain a lipid bilayer very similar to host cell membrane. The lipid bilayer and capsid inhibit penetration of acid dyes and therefore only few selected dyes have been able to stain the viral DNA/RNA. The examples of nucleic acid dyes used for labelling viral DNA/ RNA includes YOYO-1, SyBR green-I, PicoGreen, and TOTO-1 [13,14,15,16]. 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 [13,16]. After screening several commercial dyes, the membrane-permeable Syto 13 (green fluorescence) or Syto 61 (red fluorescence) works best for labeling herpes simplex virus 1 (HSV-1). Both these dyes resulted good signals, very low background noise, and excellent sample penetrance [11,12]. Most importantly, the Syto dyes are not damaging to the virion membrane at the concentration (1 µM) used therefore a plaque assay could be done for confirmation.

Labelling with fluorescent lipophilic markers in the case of enveloped viruses:
Lipid dyes are also being used for labelling of enveloped viruses for flow cytometry analysis. This approach was successfully employed for dengue virus, vaccinia virus,

HIV etc. and DiO, DiD, and DiI were used for labelling [2,17,18]. One of the most commonly used dye for labeling enveloped (animal) viruses is PKH67 which is green fluorescent molecule with an aliphatic tail that targets lipid bilayers [19,20]. SARS-VoV-2 has lipid bilayer and therefore, PKH67 could potentially be used for labelling and screening of COVID 19 cases.

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. To improve detection of the virus, in some cases, the antibodies have been pre-bound to nanobeads (15 nm) which also enhances light scattering [2, 3] during FACS analysis.

Labelling with MNPs tagged with fluorescent immunoglobulins

Qualitative and quantitative virometry analysis in flow cytometer were successfully done via application of Magnetic Nanoparticles (MNPs). 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 [2].

Apart from enumeration of viruses, flowcytometry is used in detection of viral load in PBMCs (Peripheral Blood Mononuclear Cells). Viral infections result a range of immune responses in the body and PBMCs 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 studies were done for lymphocyte subpopulation distribution in the samples of COVID 19 infected patients, and thereafter, compared the data with the cases where SARS-CoV-2 was not the cause of pneumonia [21].

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.

III. METHODOLOGY

Process and method of COVID 19 detection:

In this communication we have discussed a process of advance flow virometry to enhance testing scale of COVID 19 cases. SARS-CoV-2 belong to the family *Coronaviridae*, consisting a 29 – 30 kb chain of positive single-stranded RNA. The virus particle size ranged from 70–90 nm [22]. Extensive studies on dengue virion (40-60 nm) was done using combination of fluorescently labeled antibodies and magnetic nanoparticles (MNPs) [18]. 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. Proposed steps for sample preparation and assay are discussed in following points,

(a) Collection of oral/nasal swabs in tubes containing viral transport media, and filtering samples with a 0.45 µm cutoff membrane to minimize aggregation and artifacts in the sample.
(b) Suspension of the samples in filter sterilized ice-cold phosphate buffer saline (PBS) solution is the first step of sample preparation. Reports suggest that 1% w/v sodium azide in ice cold PBS helps to prevent the modulation and internalization of surface antigens which can help in detection process via improving fluorescence intensity of virion particles.
(c) In 1 ml of PBS suspension 0.1-10 µg of the primary antibody is added, mix the suspension and incubation the tube for 30 – 60 min at room temperature in dark. The tube could also be incubated for longer time at 4°C in the dark. Dilutions, if necessary, should be made in solution containing 3% (w/v) BSA in ice cold PBS.
(d) After incubation, the washing step is done for 3 times by centrifugation at 400 g for 5 min at 4°C. The pellet could be resuspended in ice cold PBS by gentle tapping as vigorous vortexing may reduce efficiency in detection step.
(e) Dilution of the fluorochrome-labeled secondary antibodies could be done in 3% w/v BSA in ice cold PBS (or according to the manufacturer's instructions). In 1 ml of suspended virion-antibody mix from the previous step 0.2 – 10 µg of secondary antibodies were added, and the tubes are incubated in dark for at least 20 – 30 min at room temperature.
(f) Wash the cells 3 times by centrifugation at 400 g for 5 min using 1 ml of ice-cold PBS containing 3% (w/v) BSA, 1% (w/v) sodium azide. Aspirate the supernatant using micropipette and resuspend the pellet in 100 – 200 µl of ice-cold PBS.
(g) Analysis of the cells on the flow cytometer should be done as soon as possible. We recommend that for virus studies, filtration of the sheath with 0.1 µm filter instead of 0.22 µm filter paper. Viruses are small,

therefore proper thresholds needs to be set for forward (FSC) and side scatter (SSC). 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. We propose to optimize FSC and SSC (1000 and 400) for enumeration of SARS-CoV-2.

(h) 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 [23]. The detection was made possible using fluorescein isothiocyanate (FITC)-labeled 4G2 monoclonal antibody [24]. Similarly, we expect the early detection of SAR COV-2 in suspected patients using the proposed concept.

IV. RESULTS AND DISCUSSION

Our hypothesis of screening COVID 19 samples using flow virometry could be tested in all hospitals, institutes and diagnostics equipped with BSL 2 facilities. The indirect immunofluorescence protocol discussed in this article where sequential binding of virus particles with primary and fluorescent tagged secondary antibody (specific binding to primary antibodies) would give sensitive and faster detection of SARS-CoV-2 in test samples. Outline of process flow is depicted in Fig. 1, where the test sample is incubated with the primary antibody against SARS-CoV-2, followed by secondary antibody tagged with a fluorochrome. Similar method was applied for detection of other pathogenic viruses such as dengue viruses at 3 X 10^5 particles per ml of culture [24]. Using 18 – color SORP sorter (BD FACSAria II) with 355 – 640 nm lasers, 1.0 FSC ND filter and PMT – SSC detector a flow-cytometry could detect 80 virus/ ml [25]. The primary antibody needs to be specific and can be one from human anti-SARS-CoV-2 S1 or human anti-SARS-CoV-2 spike RBD. The titration of the antibody is needed to be first determined with the positive sample dilutions. If feasible, the concept can be evaluated and limit of detection (LOD) can also be measured using cell line infected with the SARS-CoV-2 virus. In BD FACSAria II SSC detector could be positioned at the right angle to the stream and FSC around 10 -5° angle could help in improving detection process further.

Overall process of labelling the virion with the antibodies is expected to take around 90 – 120 minutes, and sample analysis in flow cytometer takes around 30 seconds. In a 96 well plate format processing of 85 - 90 samples (rest of the well is for controls could be done in 180 minutes in manual operations, however the whole process should be done in lesser time if a robotic liquid handler is added in the processing step. Therefore, in a robot assisted screening platform 1500 – 1800 samples could be screened in a day using single FACS instrument. Pooled sample analysis for scoring community spread studies will help to scale-up the analysis by 10 – 20 times per day based on design of the experiment. Overall objective to add robotic automated and liquid handler in the process is to minimize human interaction and achieve throughput.

IV. CONCLUSION AND FUTURE SCOPE

As there are not approved drug for treatment, COVID-19 has now become a major challenge for all over the world. Several plant-derived alkaloids such as chloroquine, hydroxychloroquine, bidebiline E, bisnordihydrotoxiferine, thalifaberineetc. are under studies for combating this virus [27]. Studies across globe over last few months clearly shows that the SARS-CoV-2 shows unique characteristics like: (a) highly contagious, (b) transmitted (at very low dilution) even by asymptomatic individuals, (c) the infection can be extremely severe for some individuals, forcing patients to be hospitalized and treatment in intensive care units. To detect the virus circulating within local communities, quick, sensitive and accurate detection of the infection is highly desirable. The process discussed in this article will not only help in quick and high-throughput detection of infection in densely populated habitats but also help in reducing dependencies on qRT-PCR machines and reagents. Moreover, integration of this method with robotics liquid handling devises will could also help in achieving man-less super-high-throughput sample analysis.

A global analysis of incidence of COVID 19 cases across various temperature and humidity zones by Dan *et al*. (2020) clearly indicated that variation in humidity and temperature has almost no significance in mortality caused by this virus [28]. In the first week of June 2020, the average daily rise of COVID 19 cases in India is among the top three of the World (9 – 10 thousand cases per day), which pose tremendous risk of community infection and complete disruption of healthcare system. Countries with high-end health care systems such as USA, Russia, Germany, Italy, France, UK etc. are continuously struggling to curb the infection and reduce number of fatalities. The human monoclonal antibodies that could neutralizes SARS-CoV-2 viruses were reported recently, and this may offer potential treatment of COVID 19 [29]. In countries like India where population density is very high, quick and large-scale sample analysis is highly required to curb the spread of the virus. Adoption of flow-virometry methods will also be useful for scoring community infection 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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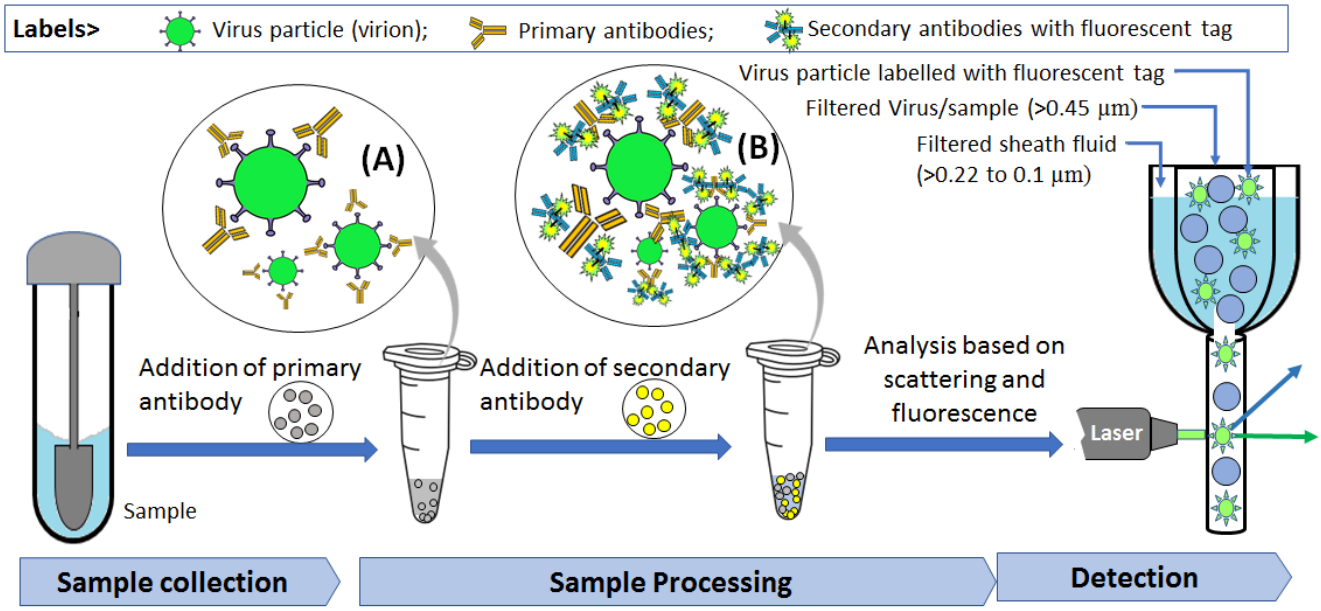

Legends to the Figure

Figure 1: The flow diagram showing steps in sample preparation and detection of SARS-CoV-2 using flow-cytometer. Test samples in viral transport media mixed with primary antibodies and then with secondary antibodies labelled with fluorescent tag. Samples were analyzed in flow cytometer using laser. (A) Picture showing binding of viral surface proteins with primary antibodies; (B) Fluorescent tagging of virus-primary antibody complex with secondary antibodies.

Table 1: Labeling and detection of viruses of different sizes using flow-virometry

Virus	Diameter	Labeling and dye	Application	References
HIV-1 virion	120-150 nm	Label enveloped viruses- lipid dyes (DiO, DiD)	HLA DR/LFA1 heterogeneity	[2]
HSV-1 virion	125 nm	Nucleic acid stain-Syto 13	Sorting of nuclear capsid	[2]
HSV-1 virion	250 nm	GFP tagging to diverse virion components	Variability of Tegument and viral fitness studies	[11]
HIV-1 virions	120-150 nm	Magnetic nanoparticles (MNPs)	Heterogeneity of HLA DR/LFA1 Studies on envelope	[12]
Dengue virus/ virion	40–60 nm	Fluorescent lipidic dye-DiI, Fluorescently labeled 2H2anti-prM antibodies, Magnetic nanoparticles (MNPs)	Studies virion maturation	[3. 18]
T4 (Bacteriophages)	70 nm X 200 nm	Nucleic acid stain-SYBR Green I	Genomics of phages	[26]

Authors Profile

Puja Pai is a Research scientist at Synthetic Biology group at Reliance Industries Ltd. She has done her M. Sc. in Biotechnology and highly skilled in flow-cytometry analysis. She has also worked on photosynthetic performance of algae and cyanobacteria and has developed methods for sorting algae cells in FACS. In addition, she is also experienced in hydroponics cultivation, indoor vertical farming and hyperspectral imaging.

Niraja Soni is a Research Scientist and Manager at Synthetic Biology group of Reliance Industries Limited. She owns Master degree in Biotechnology from Sardar Patel University, India, and highly-skilled in operation and application of FACS for sorting biological samples. She has also worked as Assistant Engineer at Flow cytometry core facility, Institute Curie, France. Her research interest includes, flow cytometry, HTP screening, feed and biomaterials.

Venkatesh Prasad is a senior scientist, heading the Synthetic Biology Function in the Reliance Industries Ltd. He has received his doctorate in Botany, from Gulbarga University. His research interests are in plant molecular biology with emphasis in crop improvement of agronomically important plants/algae, host- pathogen interactions, biofertilizers, cellular manipulations for strain improvement, genomics, cell imaging and biochemical assays.

Santanu Dasgupta is a Senior Vice President at Reliance Industries Limited. Santanu, a Ph. D in molecular biology worked on discovery of various biotech traits in the area of pathogen resistance in plants, drought tolerance and gene expression. His research also includes biofuel, food, feed, nutrition and biomaterials.

Bhaskar Bhadra is leading genome engineering function for Synthetic Biology research at Reliance Industries Limited. He has done Ph. D in chemistry and microbiology on microbe-metal interaction. He has discovered more than 10 new species of yeasts and bacteria and have worked on developing microbial cell factories for novel biochemicals and biofuel.

