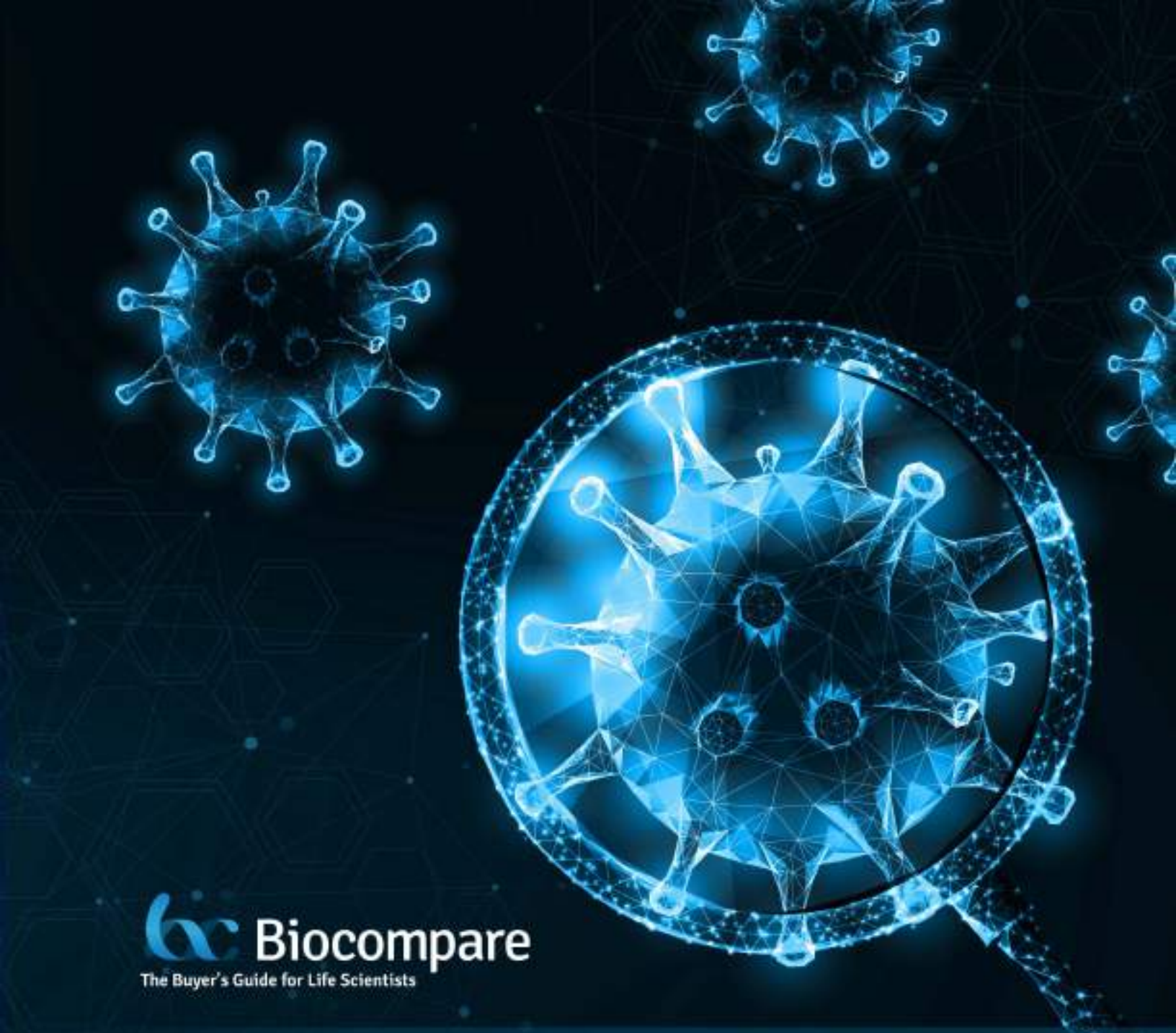

Biocompare
The Buyer's Guide for Life Scientists

Advances in Virology Research

Contents

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Tools and Technologies Advancing Virology Research

Modern scientists have an enviable toolbox to track and treat viral infections.

Although 2020 put humans in a war with SARS-CoV-2, the virus behind COVID-19, this is no new battle. Besides the influenza-driven pandemic in 1918, humans battled viral disease for centuries before that. Scientists have **showed** that humans suffered from the smallpox virus as long ago as the 6th–7th century BCE. Despite the years of contending with a variety of viral diseases, much more research must be done to provide better public health tools around the world. That work will depend on making the most of a collection of existing technologies and developing new ones.

For a useful overview of virology, watch the **Introduction to Virology, Coronaviruses, and COVID-19** from John Hopkins School of Medicine's Coronavirus Resource Center. In addition to information that is specific to COVID-19, this five-part presentation also provides useful background on virology in general.

Advances in antibodies

Decades ago, scientists started using antibodies in virology research. Even in 1982, a scientist **wrote:** "Antibodies have been used for the last five decades in the laboratory diagnosis of a wide range of diseases caused by viruses and in detailed investigations of virus structure." The use of antibodies turned especially crucial as soon as scientists detected SARS-CoV-2.

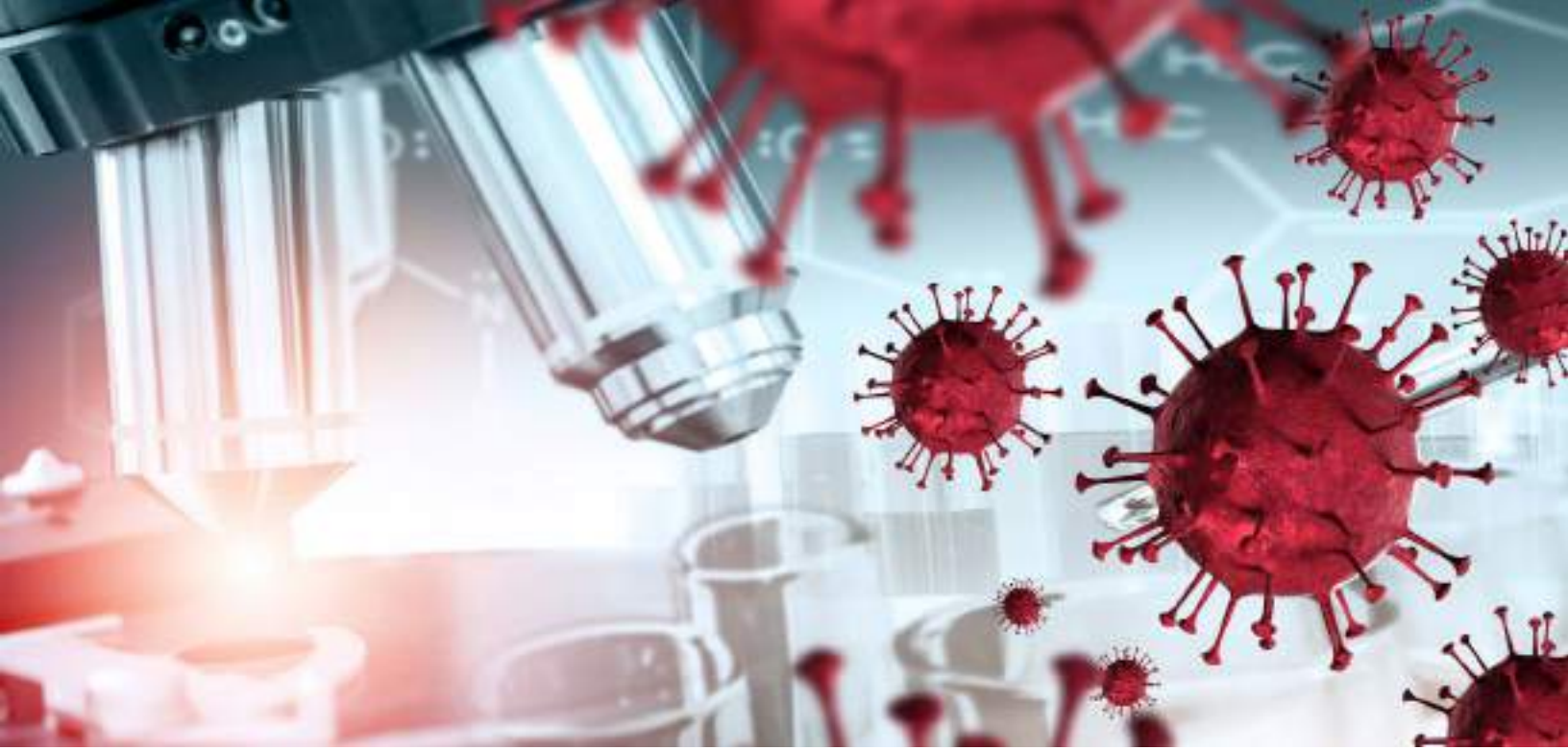
For example, some companies prepared antibodies against a range of SARS-CoV-2 antigens. Scientists can use such antibodies in experiments designed to detect the SARS-CoV-2 virus. Clinical researchers can also use these antibodies in search of mechanisms to shut down the virus.

Despite the predominance of COVID-related research in the past year, scientists also explore many other viral diseases with antibodies. As an example, one international team of scientists **discussed** the use of monoclonal antibodies as therapies for people infected with the Ebola virus.

More features with flow cytometry

To learn how a virus infects its host and replicates, scientists often rely on **flow cytometry.** This technology can be coupled with many commercial assays designed specifically for virology research.

For example, how a virus impacts a host's immune system could be studied with flow cytometry and an



assay to measure the activation or exhaustion of T cells. Plus, flow cytometry assays can be used to determine the level of antibody production triggered by the infection with a specific virus.

With live-cell analysis, other virology assays for flow cytometry can be used, such as one made to study viral infection and replication. A live-cell immunohistochemistry assay can be used with flow cytometry to measure T-cell memory and antibody titer after a viral infection—often with just a small sample.

Besides just measuring features of cells with flow cytometry, some platforms include imaging. In virology, scientists can use imaging flow cytometry to actually see changes in cells, such as morphological impacts of a viral infection. With such technology, scientists can correlate changes in markers measured through flow cytometry with differences in the appearance of cells. Consequently, data on more parameters can be collected and compared.

Expanding immunoassays

To detect proteins, scientists often use an immunoassay, such as the enzyme-linked immunosorbent assay (ELISA). Vendors offer a wide variety of ELISA-based kits. These can be used in various studies of virology. For example, some ELISA kits are designed to track specific immune-system components, such as cytokines.

Different forms of ELISA assays offer options in applications. As an example, scientists at The Tumor Hospital Affiliated to Xin Jiang Medical University in China compared a double-antigen sandwich ELISA and an indirect ELISA to diagnose hepatitis C virus (HCV). The scientists **reported** that "high agreement was found between the double-antigen sandwich ELISA and indirect ELISA," but they added that "double-antigen sandwich ELISA has distinct methodological advantages over indirect ELISA. It is recommended for the diagnosis of HCV infection."

A foundation in computation

In many areas of science—both basic research and clinical studies—large databases and sophisticated analysis play increasingly crucial roles. In fact, large datasets and advanced computational tools, such as artificial intelligence (AI), make up part of the basic

foundation of much of today's research, and virology is no exception.

A few years ago, scientists from the University of South Florida **wrote** that AI, robotics, and quality control "exemplify development of technologies in the mechanical and software realms that are accelerating advances in the defeat of animal and human infectious diseases, virology, and community medicine."

Other advanced computational methods, such as machine learning, will also be used to improve approaches to virology research. One example came from photonics expert Aydogan Ozcan of the University of California, Los Angeles, and his colleagues. These scientists developed an on-chip microscope and analyzed the three-dimensional data with deep learning-based holographic image reconstruction. Ozcan and his colleagues **wrote:** "As a proof of concept, we demonstrate rapid detection of herpes simplex virus (HSV) by monitoring the clustering of antibody-coated microparticles, achieving a detection limit of ~5 viral copies/μL."

It's too early to tell just how much AI and related forms of advanced analysis will improve virology, but it's clear that the impact could be amazing.

The changing world

Virology is all about change—lots of change. To put that in perspective, Siobain Duffy, an associate professor at the Rutgers department of ecology, evolution and natural resources, **wrote:** "RNA viruses have high mutation rates—up to a million times higher than their hosts—and these high rates are correlated with enhanced virulence and evolvability, traits considered beneficial for viruses."

Experts already knew the danger of viral evolution and infection, but the COVID-19 pandemic expanded that realization within science in general and around the world. To stay prepared for future infections, today's emphasis on virology must continue, or even accelerate.

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Advanced Tools for Virology Research

Powerful tools are helping to improve our understanding of virus biology as well as control outbreaks.

Introduction

A virus is a simple life form comprised of genetic materials—DNA or RNA—and an outer shell consisting mainly of proteins. However, even though considered an organism with only the "bare minimum" components for life, viruses are powerful pathogens and have caused several notorious pandemics that have resulted in the loss of lives and the devastation of regional as well as global economies.

Viruses require a host to survive, propagate, and change for the adaptation to environmental stresses. They interact with the host cells through the binding of virus surface proteins to their corresponding receptors and then fusion into the host cells. Once inside, the genetic materials of the viruses are released, processed, and virus propagation is achieved by hijacking various cellular machineries involved in gene transcription and protein translation (Figure 1).

Based on their unique biochemistry, pathology, and physiology, highly specialized biochemical tools are required to gain a deep understanding into the pattern of interaction between a virus and its host and to reveal the mechanisms for virus propagation and assembly. Information derived from such research helps pave the way for the development of virus diagnostic tools, public health interventions, and antiviral therapeutics. This article focuses on the development of advanced biochemical tools for virology research. These tools include recombinant virus proteins, antibodies, and pseudovirus particles.

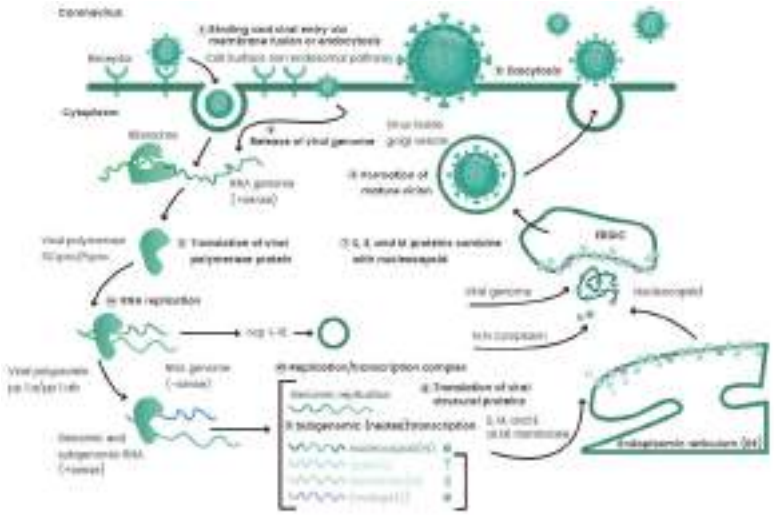

Figure 1. An example of the life cycle of a virus.

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Email: order_US@sinobiological.com

In the meantime, it is also worth mentioning that as a novel concept in vector science, certain forms of viruses are now widely used as vehicles for gene delivery and major components in vaccines. Such applications are not discussed in this article.

Recombinant virus proteins

Proteins occupy >50% (w/w) of a virus particle and they function in a variety of ways. Some proteins serve as the attachment and entry points for the virus (surface proteins, usually bear heavy glycosylations) while others catalyze the synthesis of the virus genome and processes of newly synthesized virus proteins (enzymes). These virus proteins hold the key to understanding virus physiology and also guide the generation of immunological diagnostic tools and antiviral medications. With recombinant protein expression technology, it is now feasible to produce virus proteins in large quantities and use them as tools for a variety of aspects in virology research.

In accordance with the central dogma, recombinant protein expression techniques utilize a vector (namely a plasmid) that contains the target gene encoding for the protein-of-interest and introduces it into a host cell. The target gene is either inserted into the host cell genome to guide protein translation, or the protein-of-interest is produced via plasmid-mediated direct gene translation (Figure 2). Various prokaryote and eukaryote host systems have been developed in the last four decades to meet the production of recombinant proteins with desired features in terms of post-translational modifications (PTMs), conformation, and activities (Figure 2).

The biochemistry and function of a protein dictate the selection of expression host and purification strategies. For virus surface proteins, mammalian cells are usually the preferred hosts due to their

Figure 2. Basics for recombinant protein expression and host systems.

complex high-order structures and extended glycosylation profiles, while a prokaryote system (e.g. E. coli) is usually adequate in generating active virus enzymes for both structural and functional studies. Insect cells, on the other hand, are versatile expression hosts and are used to produce both secreted and intracellular proteins. Proteins expressed by the insect system usually exert equivalent functionalities compared to those derived from the mammalian system but they bear a more simplified PTM profile to benefit structural studies. The insect cell system serves as an excellent alternative, especially for the production of hard-to-express proteins. With well-established platforms and over a decade of experience in recombinant protein expression, Sino Biological has created the largest collection of virus proteins worldwide, with more than 1,000 virus proteins covering over 100 virus strains, from popular influenza virus, coronavirus, to the more deadly filoviridae such as Ebola and Marburg virus (ProVir Viral Antigen Bank). Some examples of virus proteins are presented in Figure 3.

As an extension of conventional protein expression techniques, the transient protein expression method by HEK293 allows the establishment of

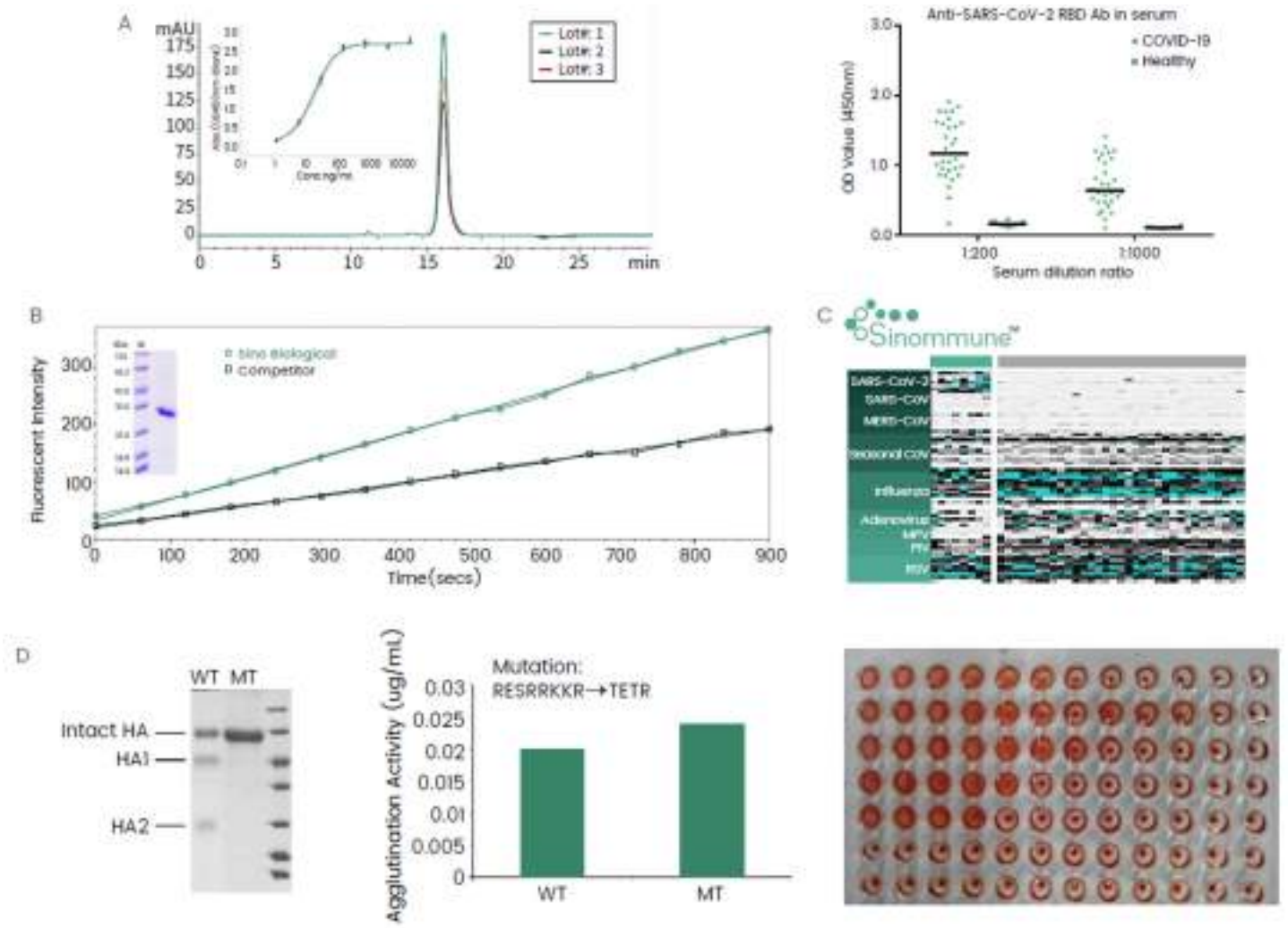

Figure 3. Examples of recombinant virus proteins from Sino Biological. (A) Receptor binding domain (RBD) of SARS-CoV-2 expressed in HEK293 cells. The protein showed good batch-to-batch consistency in terms of its purity and binding affinity against ACE2. It has been used in a serological assay to measure the serum antibody content of COVID-19 patients. (B) SARS-CoV-2 3C-like protease was expressed in insect cells with enhanced catalytic activity assayed by the cleavage of a fluorescent peptide substrate. (C) A collection of virus protein antigens for upper respiratory tract infections has been developed and formulated into the Sinommune chip, suitable for high-throughput and fast disease diagnostics. (D) Influenza (H5N1) hemagglutinin (HA) protein expressed in insect cells with agglutination activities. Mutations at the furin cleavage site abolished protein cleavage by host cells without compromising the agglutination activities.

a high-throughput protein expression platform where hundreds of constructs can be expressed simultaneously to create a recombinant protein library. This technical platform is extremely useful to cope with the high-frequency mutation rate of RNA viruses. So far, this service platform has been used to generate over 100 influenza proteins and over 80 SARS-CoV-2 RBD variants to facilitate high-throughput screening campaigns to identify broad-spectrum neutralizing antibodies.

Virus-specific antibodies and pseudovirus

When faced with a novel pathogen, the host will mount immune responses to eliminate the threat. During this process, pieces of the pathogen are recognized by immune cells followed by the generation of a pool of antibodies (immunoglobulin) to specifically "flag" the pathogen for destruction by various effector cells. With high specificities and sensitivities, virus (pathogen) specific antibodies are valuable tools for virus identification in a serological assay and neutralization, if used as a therapeutic agent. Equipped with comprehensive antibody generation platforms as well as extensive pre-existing antibody libraries, Sino Biological is highly experienced in virus-specific antibody discoveries.

For the current COVID-19 pandemic, efforts were made in both pre-existing antibody library screenings and new antibody discoveries via animal immunizations. More than 30 antibodies against the SARS-CoV-2 spike or nucleocapsid (NP) protein have been obtained, among which a few antibody pairs were identified to formulate ELISA assays with pg/mL sensitivities (Figure 4A). The NP assays have been proven to be mutation-proof when tested against NP mutants from the B1.1.7 lineage, with only a slight decrease of sensitivities observed (Figure 4C). Lastly, ultra-sensitive NP antibodies have also been identified during the screening process to boost the detection limit of NP antigen at a phantom molar level in the Simoa HD-X system (Figure 4B).

High-frequency mutagenesis is a feature of most RNA viruses. Viruses evolve through those random-

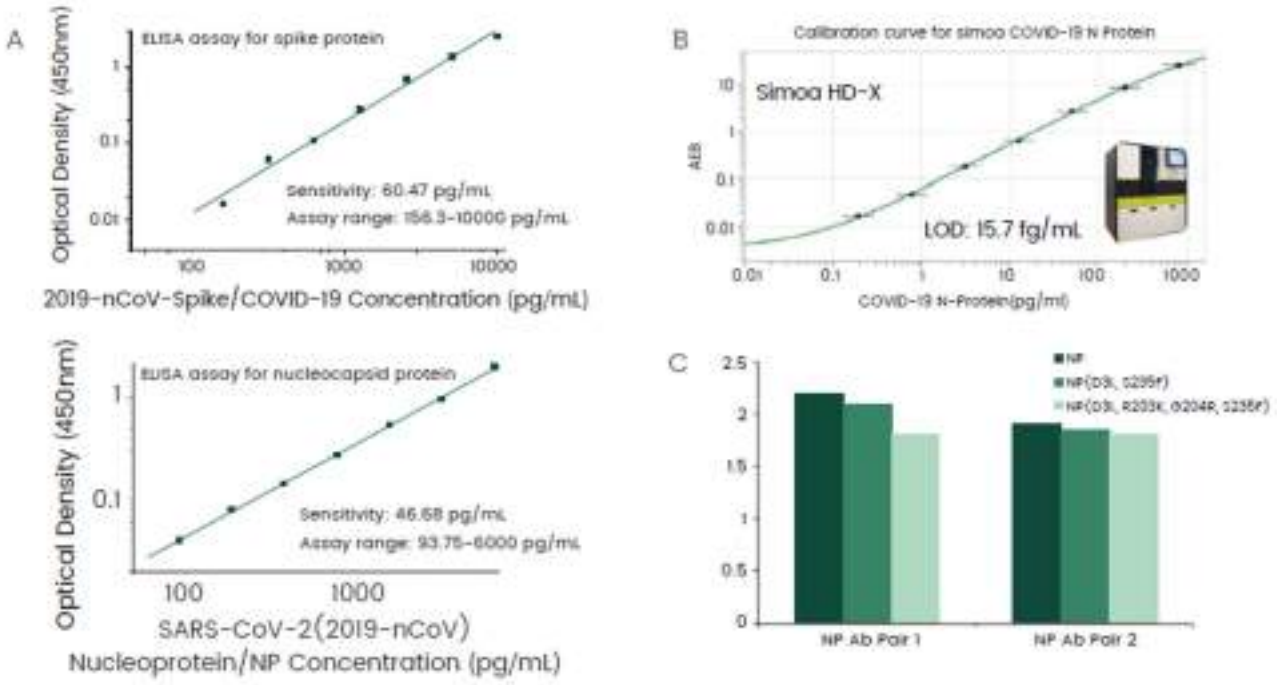

Figure 4. Highly sensitive antibody pairs for serologic assay development.

ized mutations to adapt to the new immune landscape while the mutations could be critical when occurring at key epitopes and compromise antibody efficacy significantly. The creation of a mutant protein library is useful in assessing the impact of mutations on antibody efficacy but the fluctuation of binding affinity does not directly correlate to the overall behavior of the virus.

That being said, pseudovirus is a useful tool to compensate for such shortcomings and enhance the biological relevance in the assessments of the biology of mutations. Taking advantage of current viral vector manipulation techniques, the protein-of-interest can be engineered onto the surface of the virus particles while a reporter gene is encapsulated and activated once the pseudovirus is incorporated into the host cells. The readouts of such assays are usually in the form of fluorescent intensities, and the results provide useful information to assert the virulence of a virus and assist in the investigation of the impact of mutations. One such example is presented in Figure 5.

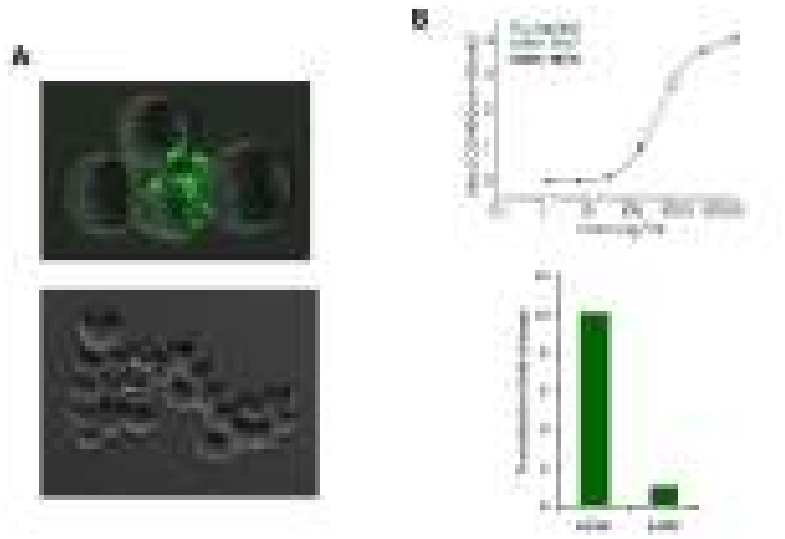

Figure 5. Application of SARS-CoV-2 pseudovirus in assessing the impact of D614G mutation. (A) A proof of concept experiment showed that the SARS-CoV-2 pseudovirus is able to bind and internalize into the host cell. Top: SARS-CoV-2 pseudovirus infected ACE2 over-expression HEK293 cell line; bottom: negative control. (B) The D614G mutation did not cause a significant alteration in ACE2 protein binding affinity judging by ELSA (top) while a SARS-CoV-2 pseudovirus containing such mutation resulted in a ~five-fold increase in the transduction fold-change, indicating a higher infectious potency given by the D614G mutation.

Conclusion

With the constant expansion of industrialization and human habitat, zoonotic viruses are lurking in the shadows, ready to emerge, and future pandemics are imminent. Recombinant virus protein, virus-specific antibodies, and pseudoviruses are powerful tools to help understand the biology of these viruses and facilitate pandemic control efforts. However, the key to pandemic prevention still lies in the rational planning of urbanization, establishment of optimized resource distribution, and exploitation of new alternative technologies to achieve the long-term and continuous development of human society.

Additional Resource

ProVir Viral Antigen Bank

Multiplex Assays to Detect Antibodies which Recognize SARS-CoV-2 Antigenic Proteins in Human Serum and Plasma

New kits allow researchers to test samples for IgG, IgA, and IgM antibodies.

Christine Kornmeier and Xiao Qiang

Introduction

The coronavirus, SARS-CoV-2, is the pathogenic agent that causes COVID-19 in humans. This disease has become a global pandemic since it was first identified in Wuhan, China, in December 2019. SARS-CoV-2 is one of seven identified coronaviruses which infect humans, and along with SARS-CoV and MERS-CoV, can cause serious disease. The remaining viral types of coronavirus (229E, NL63, OC43, HKU1) cause cold-like symptoms.

Multiple proteins make up the SARS-CoV-2 virus. The spike (S) proteins that form the "corona" of the virus are composed of the subunit S1, which contains the RBD, and subunit S2. The spikes surround the membrane glycoprotein (M) and envelope protein (E) which contain the viral RNA encased by the nucleocapsid (N) protein (Figure 1).

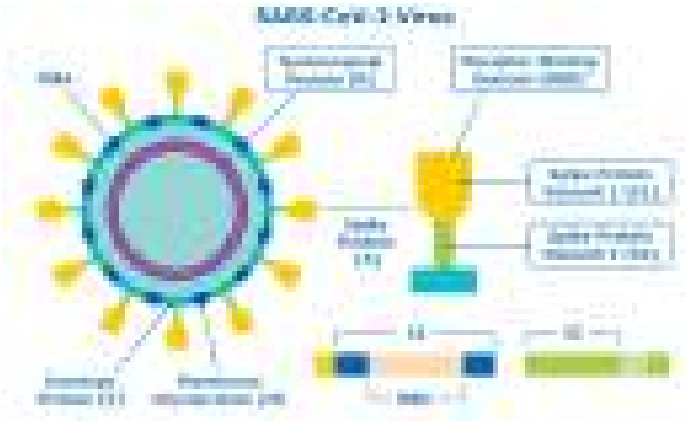

Figure 1. Antigenic proteins of the SARS-CoV-2 coronavirus.

The SARS-CoV-2 viral receptor binding domain (RBD) protein binds to the human angiotensin-converting enzyme 2 (ACE-2) receptors of cells found in multiple organs including the lungs, heart, arteries, gut, and kidneys. Once bound, the virus enters the cell, replicates, and is released to continue the infection cycle.

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Each of these viral proteins are potential antigens against which the immune system can form antibodies to fight infection. The earliest antibodies to appear are immunoglobulin A (IgA) which forms in the mucosal tissues of the nasal passages and gut, and the humoral immunoglobulin M (IgM). The humoral immunoglobulin G (IgG) forms later and can confer lasting immunity to disease. All three immunoglobulins can be measured in blood serum and plasma samples.

By testing COVID-19 patient serum/plasma sample immunoglobulin response to SARS-CoV-2 antigens, researchers may identify individuals who have been exposed to the SARS-CoV-2 virus and have generated some level of immune response. Researchers may further understand the immune response to the virus over the course of infection and recovery from COVID-19.

Multiplex Assays

We have developed three MILLIPLEX® multiplex kits for research use only (RUO), not for use in diagnostic procedures. Our MILLIPLEX® multiplex assays have been tested for use with human serum and plasma samples. Each kit can detect IgA, IgM, or IgG antibodies which recognize the selection of SARS- CoV-2 antigenic protein analytes: S1, S2, RBD, and N (Table 1). Users may select all of the analytes or just a subset of them for use. Each kit contains all reagents and the 96-well plate required to run the assay.

Table 1. MILLIPLEX® Assay Kits for COVID-19 Research.

MILLIPLEX® Assay Kits 96-well Plate Format	Cat. No.
SARS-CoV-2 Antigen Panel 1 IgM	HC19SERM1-85K
SARS-CoV-2 Antigen Panel 1 IgG	HC19SERG1-85K
SARS-CoV-2 Antigen Panel 1 IgA	HC19SERA1-85K
Available Analytes for Each Panel	
SARS-CoV-2 Spike Subunit 1 (S1)	
SARS-CoV-2 Spike Subunit 2 (S2)	
SARS-CoV-2 Receptor Binding Domain (RBD)	
SARS-CoV-2 Nucleocapsid Protein (N)	

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MILLIPLEX® assays utilize Luminex® xMAP® technology and the results in median fluorescent intensity (MFI) may be read on any Luminex® instrument system (Figure 2). The core of this technology consists of Luminex® MagPlex® magnetic carboxylated polystyrene microbeads, each dyed with a unique ratio of two fluorophores which allow the instrument to discriminate each bead with its associated bound immunoassay sandwich. The fluorophore, phycoerythrin (PE), gives the detection signal of the assay analyte.

The assay format (Figure 3) for each of the three MILLIPLEX® SARS-CoV-2 panels consists of the specific

Figure 2. Luminex® 200™, FLEXMAP 3D®, and MAGPIX® instruments.

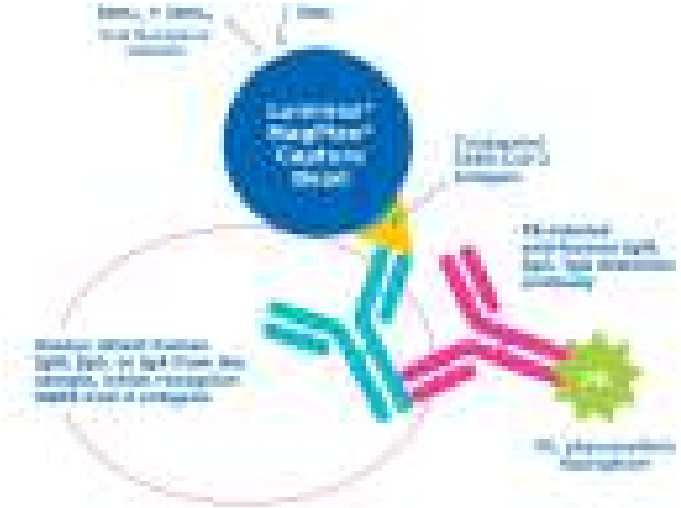

Figure 3. MILLIPLEX® assay format for these kits.

SARS-CoV-2 antigen conjugated to a unique bead region of a MagPlex® bead. These capture beads are then incubated with appropriately diluted human serum or plasma samples. Antibodies in the sample which recognize each antigen will bind, forming a bead-analyte sandwich. The sample is then washed away. A detection anti-human immunoglobulin type-specific antibody conjugated to the PE reporter is then incubated to complete the sandwich, as illustrated. Excess detection antibody is removed, and the sample MFI is read on the Luminex® instrument. Each panel is specific for the detection of human immunoglobulin IgM, IgG, or IgA. These assays are qualitative and do not include standards for quantitation. It is recommended that researchers run non-infected control samples to establish an experimental MFI cutoff.

Each kit includes a set of control beads, which may be combined in the same assay with the analyte beads, to qualify assay performance. Three immunoglobulin- conjugated Positive Assay Control beads (IgM, IgG, or IgA, dependent on the kit) have been conjugated with a different amount of immunoglobulin and will show varying levels of relative MFI readings, covering the detectable range of the assay. One Negative Assay Control bead is included (no immunoglobulin conjugation) (Figure 4).

Commercially available or in-house recombinant IgG antibodies to SARS-CoV-2 subunits S1, S2, RBD, and N were diluted into human serum/plasma collected prior to 2020 and tested in the MILLIPLEX® SARS-CoV-2 Antigen Panel 1 IgG 4-plex assay to confirm assay performance. Due to a lack of commercially available IgA and IgM antibodies to the SARS-CoV-2 analyte subunits, similar tests could not be performed on the IgA or IgM panels.

Intra-assay precision results for all three panels were found to be <15% CV as calculated from the mean of the %CVs from 8 reportable results in a single assay.

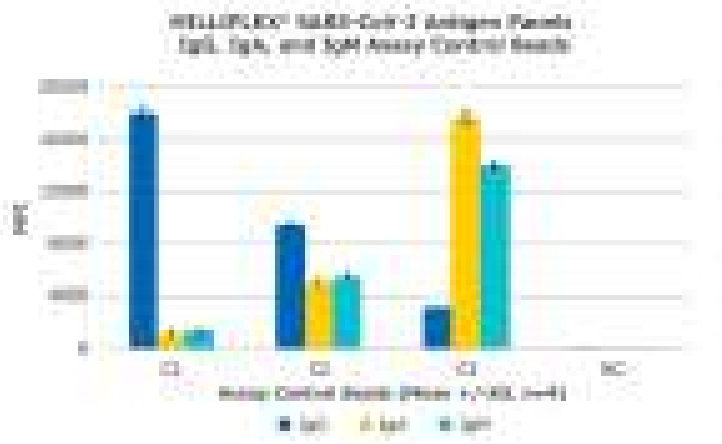

Figure 4. Assay Control Bead MFIs. Note that the IgG beads' relative MFIs read in high, medium, and low order, whereas the IgA and IgM beads' MFIs are in order of low, medium, and high relative MFI.

Inter-assay precision for all three panels were <20% CV as calculated from the mean of the %CVs across 4 different assays.

These MILLIPLEX® kits are manufactured in facilities which are ISO 9001:2015 compliant, and are for research use only, not for use in diagnostic procedures.

Materials and Methods

Samples

Patient samples were obtained, assayed, and analyzed as described.[1] The samples were from patient groups testing positive or negative by PCR for SARS-CoV-2 infection: COVID-19 positive "Ventilated" (n=68), COVID-19 positive "Not Ventilated" (n=115), and COVID-19 negative "COVID-" (n=41).

Kit Protocol

EDTA plasma samples were assayed in duplicate for all four analytes, S1, S2, RBD, and N, according to

protocol in each of the MILLIPLEX® SARS-CoV-2 kits, IgG, IgA, and IgM. All three kit protocols are identical, save for the immunoglobulin detected and the order of assay controls' MFI. Kit reagents were prepared and warmed to room temperature (RT) prior to use.

Samples were diluted 1:100 in Assay Buffer.† 96-well plate wells were pre-wetted with 200 µL Wash Buffer, covered with adhesive plate sealer, and incubated for 10 minutes at RT with shaking, then decanted. To all wells, 25 µL of Assay Buffer was added, with an additional 25 µL of Assay Buffer added to background wells. 25 µL of each diluted sample was added to the appropriate sample wells. 60 µL of each sonicated (30 seconds) and vortexed (1 minute) analyte and control bead was combined and brought to a final volume of 3 mL with the addition of Assay Buffer, vortexed, and 25 µL of bead mixture was dispensed into each well. The plate was sealed and incubated for 2 hours at RT with constant shaking to maintain bead suspension. A handheld magnetic plate washer (Cat. No. 40- 285) was used to retain magnetic beads while liquid contents were decanted and plate wells washed 3 times with 200 µL per well Wash Buffer. 50

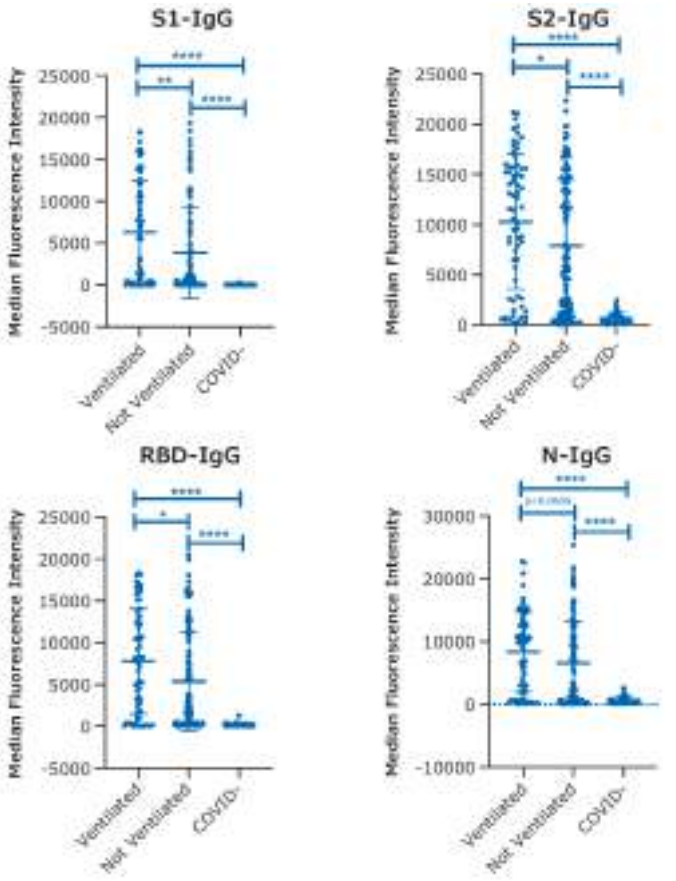

Figure 5a. Results of human patient samples run in MILLIPLEX® SARSCoV-2 Antigen Panel 1 IgG, 4-plex analytes: S1, S2, RBD, N.

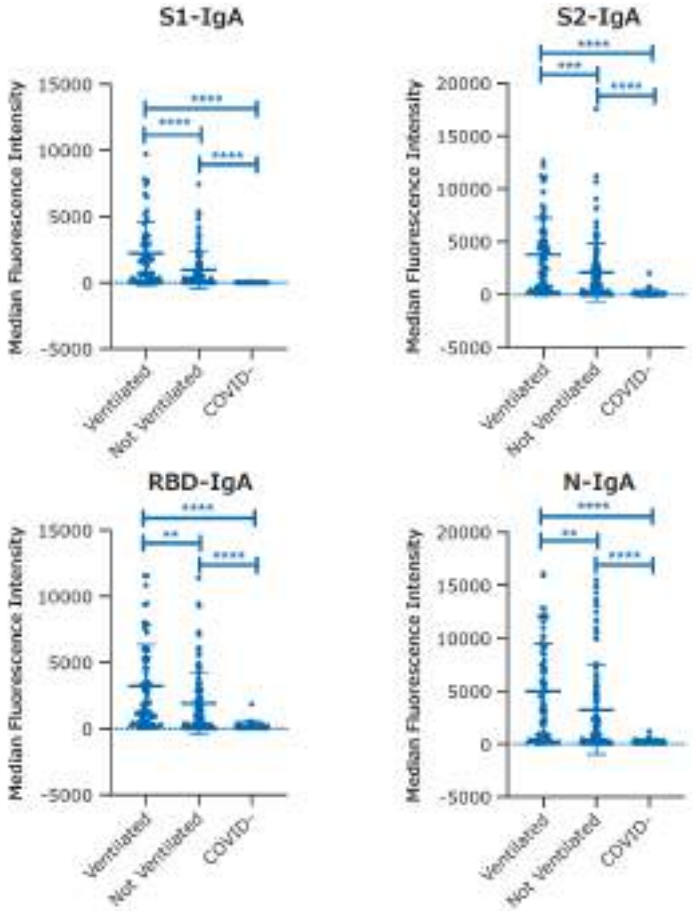

Figure 5b. Results of human patient samples run in MILLIPLEX® SARSCoV- Antigen Panel 1 IgA, 4-plex analytes: S1, S2, RBD, N.

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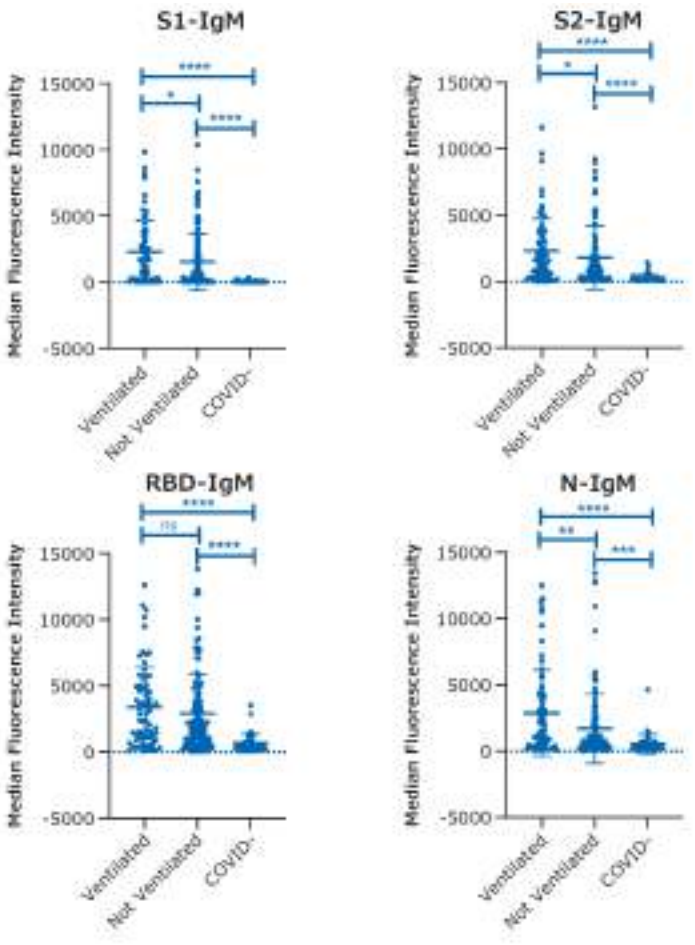

Figure 5c. Results of human patient samples run in MILLIPLEX® SARSCoV-2 Antigen Panel 1 IgM, 4-plex analytes: S1, S2, RBD, N.

µL of PE- anti-human immunoglobulin (IgG, IgA, or IgM per kit in use) detection antibody was added to each well, the plate was sealed, and incubated 90 minutes at RT with constant shaking. The magnetic plate washer was used to wash the plates 3 times with 200 µL per well Wash Buffer. 150 µL Sheath Fluid (Cat. No. 40-50015) was added to each well, the plate was then sealed and shaken at RT for 5 minutes. The plate was then read on a Luminex® 200™ instrument.

Results

Graphed assay results (Figure 5a, 5b, 5c) show individual MFI for each sample in each group with group means, +/- standard deviation (SD) and p-value significance tests between groups: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

Discussion

We have developed three new MILLIPLEX® kits which allow researchers to test human serum or plasma samples for IgG, IgA, and IgM antibodies which recognize the SARS-CoV-2 protein subunits S1, S2, RBD, and N.

These RUO assays have been verified by testing with human serum and plasma samples from a cohort of SARS-CoV-2 PCR positive and negative COVID-19 patients. These kits require minimal sample volume to run, at 25 µL of 1:100 dilution per well in a 96-well format. The assay duration is approximately one half-day, with capture-bead and sample incubation of 2 hours followed by a 90-minute incubation with detection antibody prior to reading on the required Luminex® instrument.

These kits will be of use to researchers who are studying COVID-19 in humans. Performing IgM, IgG, and IgA blood-based testing may help identify people who have been exposed to SARS-CoV-2, and who have generated some level of immune response. By testing the response of each immunoglobulin type against specific antigenic regions of the SARS-CoV-2

† Higher dilutions, such as 1:200, may be required for samples with very high antibody titer.

virus, researchers may further understand the immune response to the virus over the course of infection and recovery from COVID-19.

Now that vaccines against SARS-CoV-2 are available, these kits may provide researchers with tests to determine whether a person has immunity due to natural infection or immunity from vaccination.

Vaccinated individuals would potentially have titers against spike proteins but not the nucleocapsid protein. Of course, should vaccines containing the nucleocapsid protein as well as spike proteins come into use, these tests would no longer apply.

For additional information on these assays, contact our Technical Support team at **SigmaAldrich.com/milliplex**

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Acknowledgments

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Reference

1. IgG Antibodies against SARS-CoV-2 Correlate with Days from Symptom Onset, Viral Load and IL-10, Mary K. Young, Christine Kornmeier, Rebecca M. Carpenter, Nick R. Natale, Jennifer M. Sasson, Michael D. Solga, Amy J. Mathers, Melinda D. Poulter, Xiao Qiang, William A. Petri Jr. https://doi. org/10.1101/2020.12.05.20244541 medRxiv.org preprint dated Dec. 7, 2020.

About the authors

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Characterizing Virus Structure and Binding

Nano differential scanning calorimetry and isothermal titration calorimetry are contributing to a better understanding of the structure and mode of action of viruses.

Introduction

COVID-19 has disrupted daily global life, and major resources are focused on disease containment, treating the sick, supporting the economy, and developing treatments and vaccines. Researchers are working to understanding the basic chemical details of viral pathogenesis, viral protein structures, and identifying therapeutics to disrupt/prevent the disease. Nano Differential Scanning Calorimetry (Nano DSC) and Isothermal Titration Calorimetry (ITC) are powerful label-free and immobilization free analytic tools for thermodynamic measurements of molecular binding, biomolecular thermostability, and enzyme kinetic reactions.

The self-assembly of viral proteins and nucleic acids to form stable particles, and the mechanisms by which viruses bind to and penetrate host cells, represent two key aspects to understanding the basis of viral infection. The thermal stability of virus coat proteins in solution, compared with the stability of the corresponding highly structured viral protein shell, can be most effectively characterized by Nano DSC, whereas fundamental information about the molecular interactions driving virus/cell binding processes can be obtained by measuring the heat evolved or absorbed during binding using ITC. This note reviews applications of Nano DSC and ITC for studying the stability of virus particles and their binding to cells, as well as for the identification of viruses.

Virus Particle Stability and Assembly

Norwalk virus is the major cause of gastroenteritis. The virus consists of an RNA genome encapsulated by a coat comprised primarily of major capsid protein, and gains entry to the body through ingestion of contaminated food or water. The major capsid protein has two structurally distinct domains and self-assembles *in vitro* into empty, non-infectious virus-like particles that are structurally and immunologically similar to the intact virus. The virus experiences large variations in pH as it progresses through the infective cycle. DSC studies conducted on non-infective virus- like particles (Ausar et al., 2006) between pH 3 and 8 showed two well-separated endothermic peaks at pH 3: a larger peak centered at 66

°C and a smaller peak centered at 92 °C. At less acidic pH values, the higher temperature transition shifted to lower temperatures, and at neutral pH it was superimposed on the lower temperature transition. Proteolytic studies showed that the low temperature transition was due to the proteolytically-stable large C-terminal domain, while the high temperature transition arose from the digestible smaller N-terminal domain. These results are consistent with the need for the virus to remain intact in acidic conditions following ingestion, and to disassemble and release its RNA after penetrating the gastric cell membrane and entering the (neutral pH) cytoplasm. DSC studies on capsid-protein-only shells of a parvovirus also showed different conformations and stability in response to environmental changes similar to those encountered by the virus during its life cycle (Carreira et al., 2004). In this case, conformational changes proceeded in two steps. DSC showed that heating the shells to 46 °C caused partial externalization of the N-terminus of the protein, followed by dissociation of the shell at 75 °C into monomeric proteins.

The stabilizing effect of viral nucleic acid on virus particles has been studied using intact viruses and empty coat-protein-only shells of turnip and tobacco mosaic virus. DSC and other biophysical studies of intact turnip virus showed that heat disrupts the virus in three stages: first, the RNA is released and a conformational change occurs in the shell, then the empty shell dissociates and finally, the coat protein denatures (Mutombo et al., 1993). In tobacco mosaic virus, the intact virus is stabilized by about 15 °C compared to the coat-protein-only shell, suggesting that the RNA forms specific stabilizing interactions with the protein shell (Orlov et al., 1998). The thermal stability of the coat protein alone in solution or in small aggregates is about 30 °C lower than when packed into the highly structured coat-protein-only shell. The authors point out that the presence of folded coat protein in solution is known to inhibit virus disassembly. This would aid infection, as the instability of monomeric or small coat protein aggregates would help drive the disassembly process to completion once initiated by the release and unfolding of a few coat protein molecules in the infected cell.

DSC has also been used to investigate the thermal stability of adenovirus, a favored vehicle for delivering transgenic material to somatic cells, with the intent of identifying the effects of environmental factors such as pH and sugars on virus stability (Rexroad et al., 2002; Ihnat et al., 2005). Ihnat et al. (2005) studied the purified wildtype major capsid protein (hexon protein), the wildtype virus (Ad/WT) and a mutant form of the virus (rAd/p53) in various buffers. The mutant virus exhibited three endothermic transitions, while the wildtype provided four transitions. The coat protein provided two endotherms (Fig A) but lacked the 50 °C endotherm characteristic of the intact virus. Deconvolution of the approx. 50 °C peak (Fig B) from the virus particles showed that this endotherm was composed of two thermal events in a concentration-dependent manner. Taken together with other calorimetric data, the authors conclude that the 50 °C transition represents the disruption of the virus particle into protein and nucleic acid, and that the transitions at approx. 70 and 75 °C arise from conformational changes in the hexon protein. The 50 °C transition is not reversible and since its position, size and shape are dependent on concentration and environment, the authors suggest that the position of this transition can be used to distinguish between adenovirus mutants, and to screen and optimize stabilizing formulations.

The assembly of complex viruses such as bacteriophages and some eukaryotic viruses often requires the assistance of chaperones and proteases. The thermal stability of viruses has long been studied by DSC, and changes in thermal stability during the

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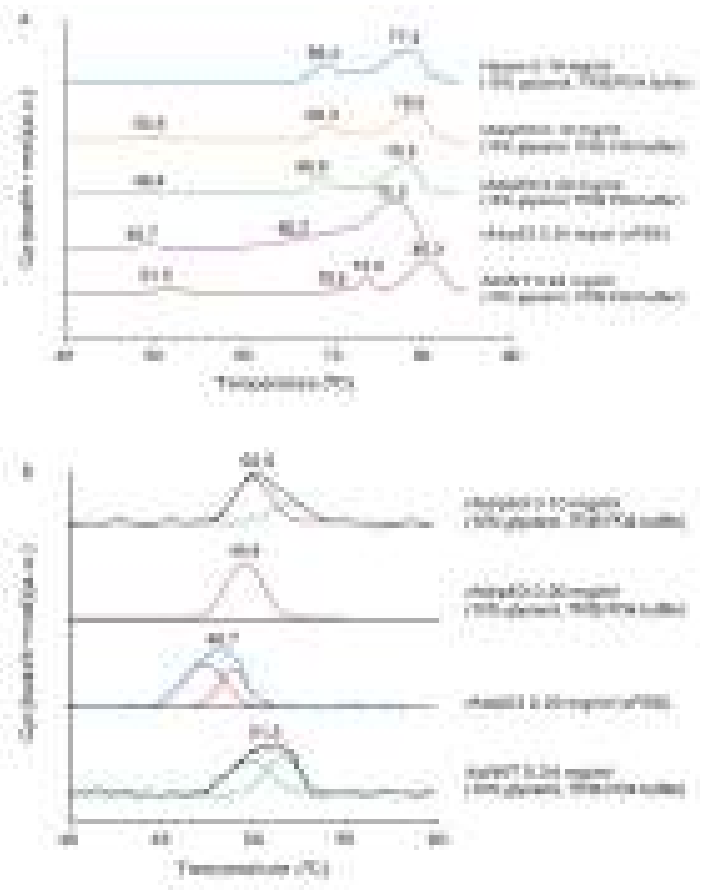

Figure 1A. DSC scans of capsid protein alone (hexon), mutant virus (rAd/p53) at two concentrations and in different buffers, and wildtype virus (Ad/WT). Samples were scanned at 1 °C/min in a Nano-DSC.

B. Result of deconvolution of ~ 50 °C peak. (Ihnat et al., 2005).

maturation process have been correlated to structural changes observed by X-ray scattering and electron microscopy. Perhaps the most detailed thermal profiles have been obtained for the bacteriophages T4 (Ross et al., 1985; Steven et al., 1992), P22 (Galisteo and King, 1993; Galisteo et al., 1995), and HK97 (Ross et al., 2005; Ross et al., 2006). In the case of HK97, the precursor capsid protein, gp5, self-assembles into pentamers and hexamers which in turn self-assemble into procapsids. This is followed by N-terminal proteolysis, expansion of the capsid particle, conformational rearrangements, covalent cross-linking, and insertion of the DNA (Ross et al., 2006). The maturation process results in progressively more stable particles, and some steps (proteolysis and cross-linking) prevent reversal of the process. The intact virus cannot be thermally disassembled without the use of 5 M urea.

Virus Entry Into Cells

Viruses enter cells through one or a combination of two general mechanisms: direct fusion with the cell membrane (enveloped viruses) and entry via endocytotic or other vesicles (non-enveloped viruses). The latter mechanism is mediated by cell surface receptors, although some viruses may fuse with the host cell membrane in the absence of a receptor. Calorimetry has been used to study both types of infection pathways.

Enveloped animal viruses enter the host cell by fusing with the host cell membrane, induced by conformational changes in viral coat glycoproteins. The conformational change can be triggered by the acidic environment of the endosome, or by interaction with a specific receptor on the cell surface. Influenza virus fuses with cell membranes under acidic conditions. The virus coat consists of a lipid membrane and three transmembrane proteins of which one, hemagglutinin, undergoes a large conformational change at acidic pH. This exposes a hydrophobic segment that drives virus fusion with the host cell membrane. DSC of purified hemagglutinin at neutral pH produces a single large-enthalpy cooperative endotherm with a T_m of 66 °C, whereas at pH 5 the protein unfolds in a less cooperative manner at 45 °C, with approximately one-third the enthalpy change (Remeta et al., 2002). DSC and spectroscopic studies on intact influenza virus, conducted to de-

termine how the hemagglutinin protein responds to pH and temperature when embedded in the viral membrane, also showed significant differences between acidic and neutral pH (Epand and Epand, 2002). As with the purified protein, hemagglutinin in the intact virus unfolded cooperatively at about 66 °C at neutral pH, but at pH 5, the T_m decreased only to 60 °C and the enthalpy remained essentially unchanged, although cooperativity was significantly reduced. The authors concluded that the decreased stability of hemagglutinin at acidic pH initiates virus/cell membrane fusion mediated by the exposed hydrophobic segment, but that hemagglitinin within the intact virus is less destabilized than the purified protein under similar conditions. ITC experiments with intact and inactivated influenza virus at neutral and acid pH allowed binding and membrane fusion events to be separated and showed that membrane fusion is an endothermic reaction occurring exclusively at acidic pH (Nebel et al., 1995).

ITC and other studies on vesicular stomatitis virus showed that the viral fusion protein bound to and fused with phosphatidyl choline/ phosphatidyl serine (1:3) vesicles exothermally at pH 6.0, whereas binding but no fusion occurred at pH 7.5 (Carneiro et al., 2002, 2006). The fact that binding was exothermic suggested that it might be driven by electrostatic interactions. No binding was observed when the vesicles did not contain phosphatidyl serine, or when the histidines in peptides corresponding to the putative binding region on the viral fusion protein were replaced with alanines. The ITC data suggest that binding of the viral fusion protein to the cell brings histidine residues in the binding region of the protein in close proximity with the negatively charged phosphatidyl serine head groups, leading to protonation of the histidines and interaction of the fusion protein with the cell membrane.

HIV is perhaps the most extensively studied enveloped virus. Infection is initiated when the viral coat glycoprotein, gp120, interacts with CD4 receptors on the target cell, triggering conformational changes in the virus envelope and leading to fusion with the target cell. ITC studies of the interaction between gp120 and CD4 (Myszka et al., 2000) showed a very favorable binding enthalpy (approx. -63 kcal/mol (-264 kJ/mol)), indicating the formation of a large number of hydrogen bonds and van der Waals interactions. However, the binding entropy is large and unfavorable, indicating significant loss of conformational freedom upon binding and resulting in medium strength ($2 \times 10^8\ M^{-1}$) binding. In addition, the binding reaction is slow. Independent studies on the CD4 protein showed that its structure remains essentially unchanged upon binding, demonstrating that the unfavorable entropy arises primarily from structural rearrangement of gp120. Slow binding is consistent with significant conformational changes occurring during complex formation.

Extensive studies on a nonenveloped virus, simian virus 40, has shed light on how a virus recognizes and binds to its receptor on the host cell, without binding so tightly that replicated viruses cannot migrate to new host cells. ITC data showed that the virus recognizes its glycoprotein receptor with millimolar affinity, but glycan screening experiments showed that when presented with an array of glycans, the virus binds preferentially to the branched oligosaccharide found on the receptor. Crystallographic data show that binding specificity is achieved by the two branches of the oligosaccharide interacting simultaneously with the binding cleft (Neu et al., 2008).

Identifying Viruses by DSC

In 1997, Shnyrov et al. showed that scanning a Newcastle disease virus sample to successively higher

temperatures on DSC (successive annealing) provided four endothermic two- state transitions which corresponded to the four major proteins in the virus. The results suggested that the DSC profile of a virus could be used as a means of identification. This concept has been extended beyond the identification of the species of virus, to the identification of individual strains. For example, Krell et al. (2005) used DSC to characterize various polio and influenza virus strains. Each strain had a characteristic pattern of multiple unfolding transitions which differed in T_m, shape and enthalpy, with significant pattern differences even between closely-related strains (e.g., the highly-related H1N1 influenza strains A/PR8/34 and New Caledonia, or the H3N2 strains X-31 and Panama). The authors suggest that the surface coat proteins strongly influence the DSC profile of the virus. Since it is these proteins that often differ between closely related strains, DSC could be a widely-applicable, simple and fast clinical approach for identifying infective viral strains.

Summary

The body of research incorporating DSC and ITC studies for understanding the structure and mode of action of viruses is growing rapidly. DSC is invaluable for elucidating the thermodynamic profile of a virus structure, leading to an understanding of the stability of viral components and the reliable characterization and identification of virus strains. Virus virulence depends on the ability of the virus to specifically bind to its target and infect the cells. ITC has been shown to be a robust assay technology that can be used to effectively characterize any inter-molecular binding events occurring between a virus and its receptors. Because viruses are composed of only a few major protein and nucleic acid components, it is perhaps not surprising that details of how these components pack and interact with each other can be studied calorimetrically using intact viruses. At the same time, the level of specific structural information from a well-designed DSC or ITC experiment conducted on intact viruses can rival that of other techniques that require purified protein or nucleic acid components to provide interpretable data. Rapid accurate evaluation of viral structure and virulence factors could be critical for expanding the structure-activity relationship database for viruses, thereby allowing for a timely response to viral outbreaks and pandemics.

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Lentivirus-Based SARS-CoV-2 Virus Particles for the Study of COVID-19

Off-the-shelf particles help elucidate the mechanisms behind SARS-CoV-2 entry into cells.

Background

Emerging viruses are a global threat to human health and economic stability, as exemplified by the current COVID-19 pandemic caused by novel coronavirus SARS-CoV-2, which is efficiently transmitted from human to human. COVID-19 has spread rapidly, creating a global emergency situation. The scientific community reacted swiftly and massively worldwide, supplying diagnostic tests, generating new therapies, and designing much needed vaccines.

Scientists at BPS Bioscience have been actively working on developing COVID-19 research tools to support the scientific community in these efforts. These research tools include an entirely new line of lentiviruses to study the first step of SARS-CoV-2 infection.

The Spike protein consists of two subunits (S1 and S2) organized in a homo-trimer structure. SARS-CoV-2 infects human cells when the RBD (Receptor Binding Domain) region of the Spike S1 subunit binds to ACE2 (angiotensin converting enzyme 2) on the surface of cells in the respiratory system including the lungs, in the arteries, heart, kidney, and intestines. Based on this observation, drugs or neutralizing antibodies that target the interaction between the Spike protein of SARS-CoV-2 and human ACE2 may offer protection against viral infection. Spike then needs to be primed by extracellular proteases Furin and TMPRSS2 (human transmembrane serine protease 2), primarily expressed by endothelial cells across the respiratory and digestive tracts. These proteases cleave Spike at

Figure 1. SARS-CoV-2

DOUBLING DOWN ON SARS-COV-2 EMERGING VARIANTS

BPS has developed a collection of recombinant proteins, unique assay kits, pseudovirions, antibodies, and lentiviruses to help intensify research of emergeng variants and their effects on pathogenesis, current therapeutic drugs,and vaccine development.

Pseudotyped Lentiviral SARS-CoV-2 Variants

PRODUCT	NAME
Pseudotyped Lentivirus	Spike (P.1 Variant) (SARS-CoV-2) Pseudotyped Lentivirus (Luc Reporter)
Pseudotyped Lentivirus	Spike (B.1.351 Variant) (SARS-CoV-2) Pseudotyped Lentivirus (Luc Reporter)
Pseudotyped Lentivirus	Spike (B.1.1.7 Variant) (SARS-CoV-2) Pseudotyped Lentivirus (Luc Reporter)
	+ MORE

Recombinant SARS-CoV-2 Variant Proteins

PRODUCT	NAME
Protein	Spike Trimer (S1+S2) (P.1 Variant), His-Tag (SARS-CoV-2)
Protein	Spike RBD (B.1.351) Avi-His-Tag (SARS-CoV-2)
Protein	Spike Trimer (S1+S2) (B.1.351 Variant), His-Tag (SARS-CoV-2)
Protein	Spike RBD (B.1.1.7), Avi-His-Tag (SARS-CoV-2)
Protein	Spike Trimer (S1+S2) (B.1.1.7 Variant), His-Tag (SARS-CoV-2)
Protein	Nucleocapsid Protein (B.1.351 Variant), Avi-His-Tag (SARS-CoV-2)
	+ MORE

SARS-CoV-2 Variant Inhibitor Screening Assay Kits

PRODUCT	NAME
Assay Kit	Spike S1 RBD (B.1.351 Variant) (SARS-CoV-2): ACE2 Inhibitor Screening Chemiluminescence Assay Kit
Assay Kit	Spike RBD (B.1.1.7 Variant) (N501Y) (SARS-CoV-2): ACE2 Inhibitor Screening Colorimetric Assay Kit
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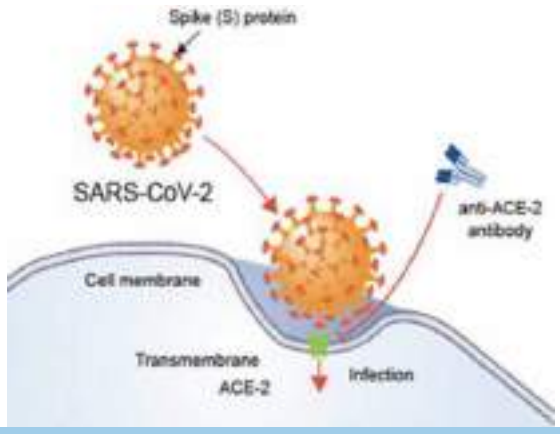


Figure 2. SARS-CoV-2 virus entry into host cells

the S1/S2 site, exposing a peptide in the S2 subunit that promotes virus fusion with the cellular membrane, thereby allowing the virion to enter the cell. Since TMPRSS2 is involved in viral entry, it has been proposed that blocking TMPRSS2 activity could also be an effective therapeutic strategy.

The emergence of SARS-CoV-2 mutants is a serious threat, particularly when new mutations result in higher transmissibility and infectivity. Thousands of variants have now been described, with notable variants of concern: Variant (B.1.1.7) identified in the United Kingdom, Variant (B.1.351) identified in South Africa, or Variant (P.1) identified in Brazil. Fast-spreading Variant B.1.1.7, for example, contains several mutations including a N501Y mutation that is thought to make it easier for the virus to attach to human ACE2. Research on these variants will be critical to update vaccines and therapeutics.

Lentivirus-based Tools

To better understand the mechanism of SARS-CoV-2 entry into cells, BPS Bioscience has developed new, off-the-shelf lentivirus-based virus particles that can infect most types of mammalian cells, including primary and non-dividing cells. These viruses are ready for infection and do not require other components. Infection rates as high as 90% and efficient gene transduction lead to robust expression of proteins of interest or reporter proteins. Importantly, none of the HIV genes (gag, pol, rev) are expressed in the infected cells, therefore our lentiviruses are replication-incompetent and can be used in a Biosafety Level 2 facility, which makes them accessible to many research laboratories.

Our first family of lentiviruses for COVID-19 research are replication incompetent, pseudotyped lentiviral particles that use protein VSV-G (Vesicular stomatitis virus G protein) as an envelope protein to mediate cellular entry via the LDL receptor (low-density lipoprotein receptor), allowing infection of various cell types. These viruses are used to drive transient or stable expression of proteins of interest ACE2 (BPS Bioscience #79944), TMPRSS2 (BPS Bioscience #78011), and Spike (BPS Bioscience #78010).

Figure 3 illustrates the structure of the ACE2 vector used to transduce the gene encoding ACE2, while

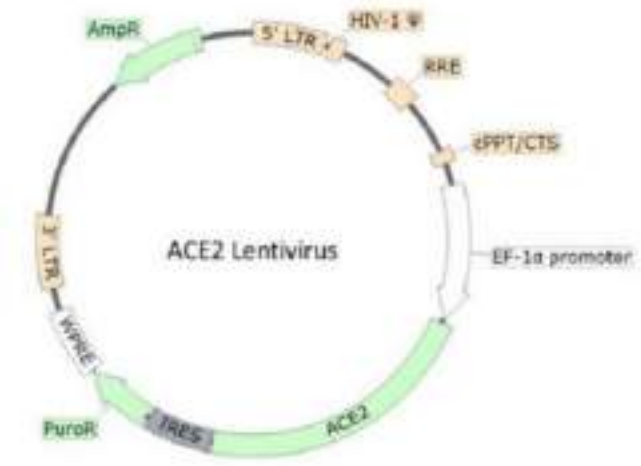


Figure 3. ACE2 lentivirus construct. Under the control of EF1a promoter, the virion transduces ACE2 (NM_021804.3) allowing its transient expression in target cells or the generation of a stable cell line expressing ACE2 (following puromycin selection).

Figure 4 shows ACE2 expression following infection of HEK293 cells with ACE2 Lentivirus (BPS Bioscience #79944) measured by flow cytometry.

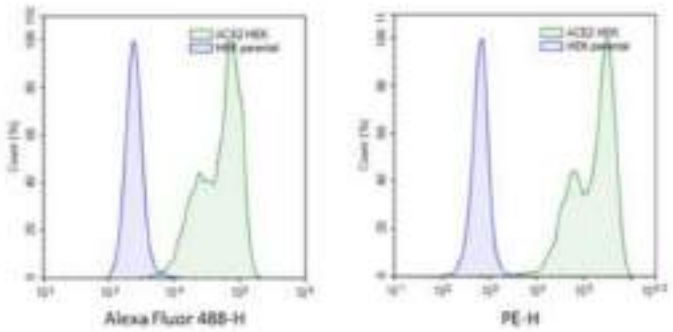


Figure 4. Flow cytometry analysis of ACE2 expression in HEK293 cells transduced with ACE2 Lentivirus (BPS Bioscience #79944). Blue: HEK293 parental cells; Green: HEK293 cells transduced with ACE2 lentivirus (left panel: Alexa Fluor 488; right panel: PE)

Pseudotyped SARS-CoV-2 Lentiviruses

Our second generation off-the-shelf pseudotyped lentiviruses are designed to study the interaction between the SARS-CoV-2 Spike protein and human ACE2 in a physiologically relevant context. These viruses use SARS-CoV-2 Spike protein (GenBank #QHD43416.1) as the envelope protein to infect COVID-19-relevant target cells. They drive the expression of Firefly luciferase for precise and robust quantification of infection, enhanced GFP (eGFP) for fluorescence-based experiments, or both. For example, Spike SARS-CoV-2 Pseudotyped Lentivirus Luciferase reporter (BPS Bioscience #79942) contains the vector shown on the left in Figure 5. Spike SARS-CoV-2 Pseudotyped Lentivirus eGFP corresponds to BPS Bioscience #79981 (not shown), and pike SARS-CoV-2 Pseudotyped Lentivirus dual reporter (BPS Bioscience #79982) contains the vector shown on the right in Figure 5.

These pseudotyped lentiviruses are particularly useful to measure the effect of neutralizing antibodies on Spike-mediated infectivity and gene transduction, as shown in Figure 6.

To serve as negative controls for this family of reporter pseudoviruses, bald lentiviruses were designed

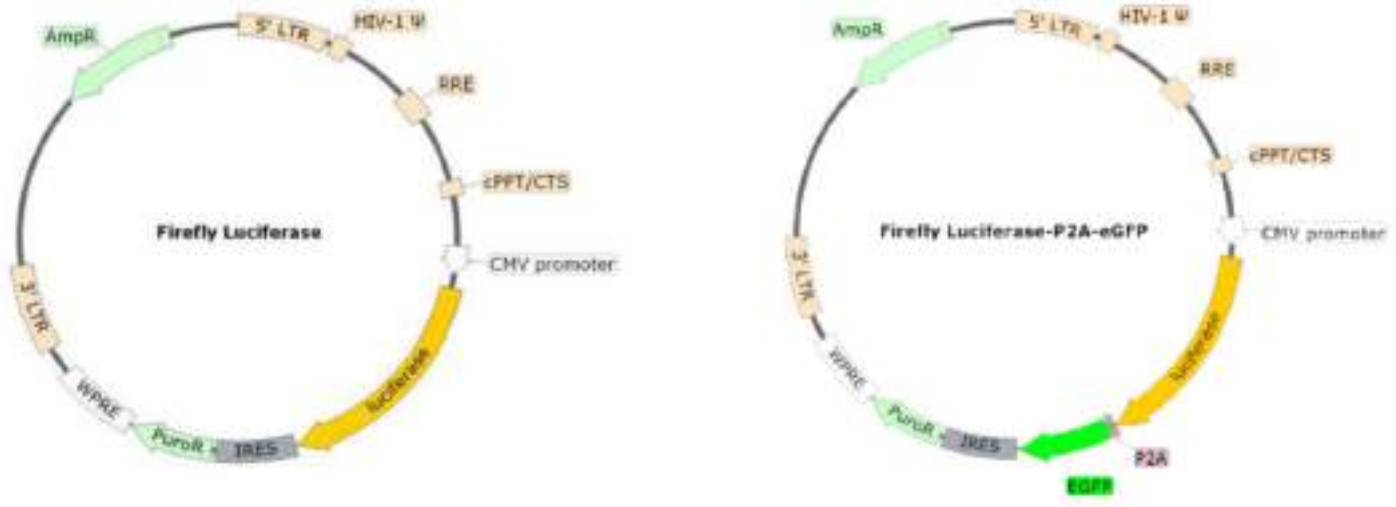


Figure 5. Spike SARS-CoV-2 Pseudotyped Lentivirus Luc Reporter (BPS Bioscience #79942) and Dual Reporter (BPS Bioscience #79982).

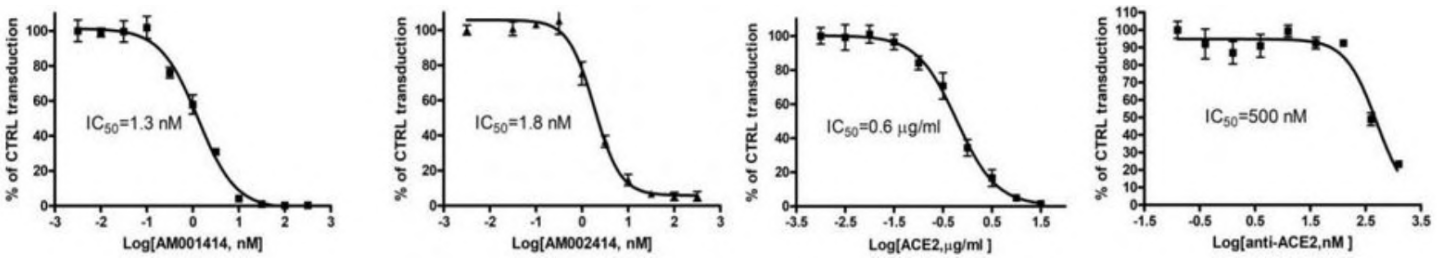


Figure 6. Neutralization assays performed with two distinct anti-SARS-CoV-2 Spike antibodies, a recombinant ACE2 protein, and an anti-ACE2 antibody (from left to right). HEK293 cells expressing ACE2 were transduced with a mix containing the Spike (SARS-CoV-2) pseudotyped lentivirus and different concentrations of the corresponding neutralizing compounds. Control transduction (100%) corresponds to the luciferase activity measured in presence of the virus without neutralizing compounds. Three independent experiments showed dose-dependent decreases in the transduction efficiency of the Spike (SARS-CoV-2) Pseudotyped Lentivirus due to neutralizing effects of the tested compounds.

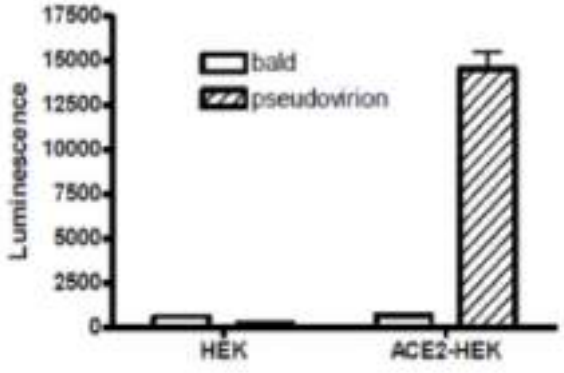


Figure 7. Infection of ACE2-HEK293 cells monitored by luciferase activity. HEK293 cells were infected with ACE2 Lentivirus or with Bald Lentivirus Luc-eGFP Dual Reporter (BPS Bioscience #79988).

without envelope glycoprotein (neither VSV-G nor SARS-CoV-2 Spike) so they cannot infect cells, but contain the firefly luciferase gene and/or eGFP: Bald Lentiviral Pseudovirion Luciferase Reporter (BPS Bioscience #79943), eGFP Reporter (BPS Bioscience #79987), and Luc-eGFP Dual Reporter (BPS Bioscience #79988).

SARS-CoV-2 Variants

Mutant viruses are available to study emerging SARS-CoV-2 variants and to measure the effect of select mutations on the interaction between Spike and ACE2 on virus infectivity. Currently available variant viruses include Spike SARS-CoV-2 Pseudotyped Lentivirus Luc Reporter B.1.1.7 Variant, identified in the UK (BPS Bioscience #78112) and B.1.351 Variant, identified in South Africa (BPS Bioscience #78142), while Variant P.1 (first identified in Brazil) is in the pipeline. Mutant Spike pseudotyped viruses containing only the highly conserved D614G mutation

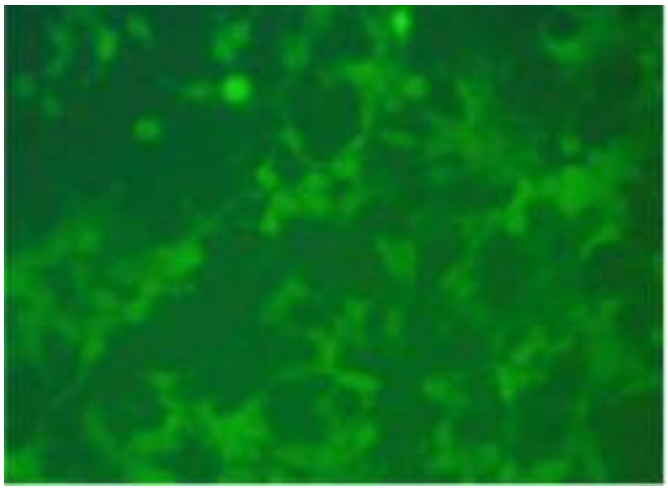


Figure 8. Infection of ACE2-HEK293 cells with SARS-CoV-2 Spike (D614G) pseudotyped lentivirus eGFP reporter (BPS Bioscience #78035).

(eGFP reporter BPS Bioscience #78035 or Luc reporter BPS Bioscience #78028) or the 3 mutations K417T, E484K, N501Y present in the RBD (Receptor Binding Domain) of the P.1 variant (Luc reporter BPS Bioscience #78143) have also been released, with more to come. These mutant pseudoviruses can be useful for differentiating the binding site and mechanism of action of neutralizing antibodies. Figure 8 shows an example in which cell entry of Spike mutant (D614G) was visualized using eGFP fluorescence.

Finally, our study shown in Figure 9 compared the effect of anti-Spike antibodies on the infectivity of wild-type and Variant B.1.1.7 Spike-pseudotyped viruses in ACE2-HEK293 cells. It was observed that the wild-type virus was neutralized by this antibody in a dose-dependent fashion (left panel), whereas Variant B.1.1.7 was not (right panel).

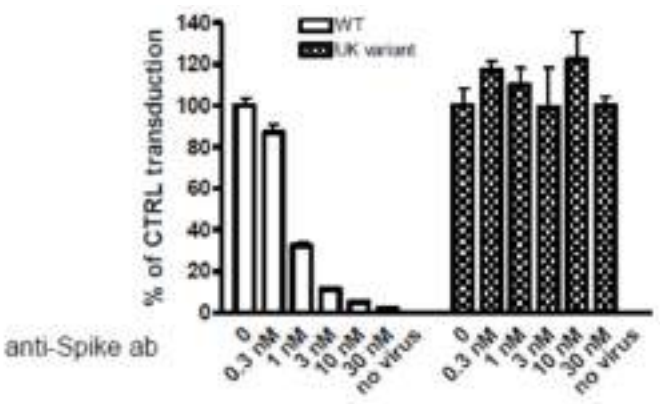


Figure 9. Effect of anti-Spike antibodies on the transduction efficiency of B.1.1.7 Variant (UK) compared to wild-type (WT) Spike. ACE2- HEK293 cells (BPS Bioscience #79951) were infected with Spike Pseudotyped Lentivirus Luciferase Reporter (BPS Bioscience #79942) or with Spike B.1.1.7 Variant Pseudotyped Lentivirus Luciferase Reporter (BPS Bioscience #78112) in the presence of increasing concentrations of an anti-Spike antibody. Control transduction (100%) corresponds to luciferase activity measured in the presence of WT virus without neutralizing antibody.

Conclusion

BPS Bioscience has recognized the urgency and special challenges of conducting coronavirus research during the pandemic. BPS scientists are continually striving to offer novel and innovative products and services to help researchers design new therapeutics and vaccines for COVID-19. We have developed one of the largest portfolios of coronavirus research tools and services. These include cell lines expressing ACE2 and TMPRSS2, unique assays to measure the activity of 3CL, PLPro, TMPRSS2, Furin, RdRP, and other key SARS-CoV-2 targets, and a rapidly expanding portfolio of lentivirus-based virus particles to keep pace with the emergence of new variants of concern and facilitate the study of these new mutations on virus biology, diagnostic test performance (serology or antigen tests) and vaccine efficacy.

Additional Resource

Coronavirus Protein, Kits, Lentiviruses, Antibodies, Custom Services

What We Know about SARS-CoV-2 Infectivity and Growing Concerns with Emerging Variants

Rapid progress has been made, but a better understanding of emerging mutations is key to turning the tide on this pandemic.

Spreading rapidly worldwide, COVID-19 is recognized as the most severe global public health emergency in history by the World Health Organization. COVID-19 has become a huge threat to human health, safety, and global economics. In addition, the continuing pandemic poses a real challenge to global health governance and human sustainability.

However, rapid advancement in understanding the structural underpinning of SARS-CoV-2 infectivity and transmissibility has yielded promising therapeutics and vaccine candidates. Continual examination and study of viral mechanisms and the impact of emerging mutations are key to turning the tide on this pandemic.

Understanding infectivity

The SARS-CoV-2 viral particle consists of a lipid bilayer containing membrane (M) protein, envelope (E) protein, nucleocapsid (N) protein, and the Spike (S) protein as shown in Figure 1.

Prior to the SARS-CoV-2 outbreak, the S protein of SARS-CoV had been studied extensively. The S protein, among other structural proteins of SARS-CoV, was found to be highly immunogenic and effective neutralizing antibodies recovered from SARS patients were directed at S protein.[1,2] Therefore, research into vaccines and therapeutics against SARS-CoV-2 were immediately focused on the S protein.[3]

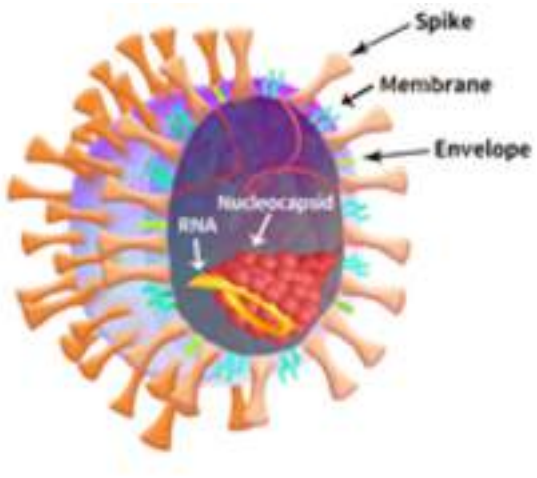


Figure 1. SARS-CoV-2 particle schematic.

The S1 subunit contains a NTD and a RBD that binds to the human ACE2 primarily through direct interaction with the receptor binding motif (RBM). The

Critical Reagents for SARS-CoV-2 Research

Diagnostics · Vaccines · Therapeutics

120+ Structural proteins

10+ Non-structural proteins

60+ Mutants

10+ Other coronavirus proteins

40+ Antibodies

20+ Kits

10+ Pre-coupled beads

60+ mutants from dominant SARS-CoV-2 variants

All critical mutations covered

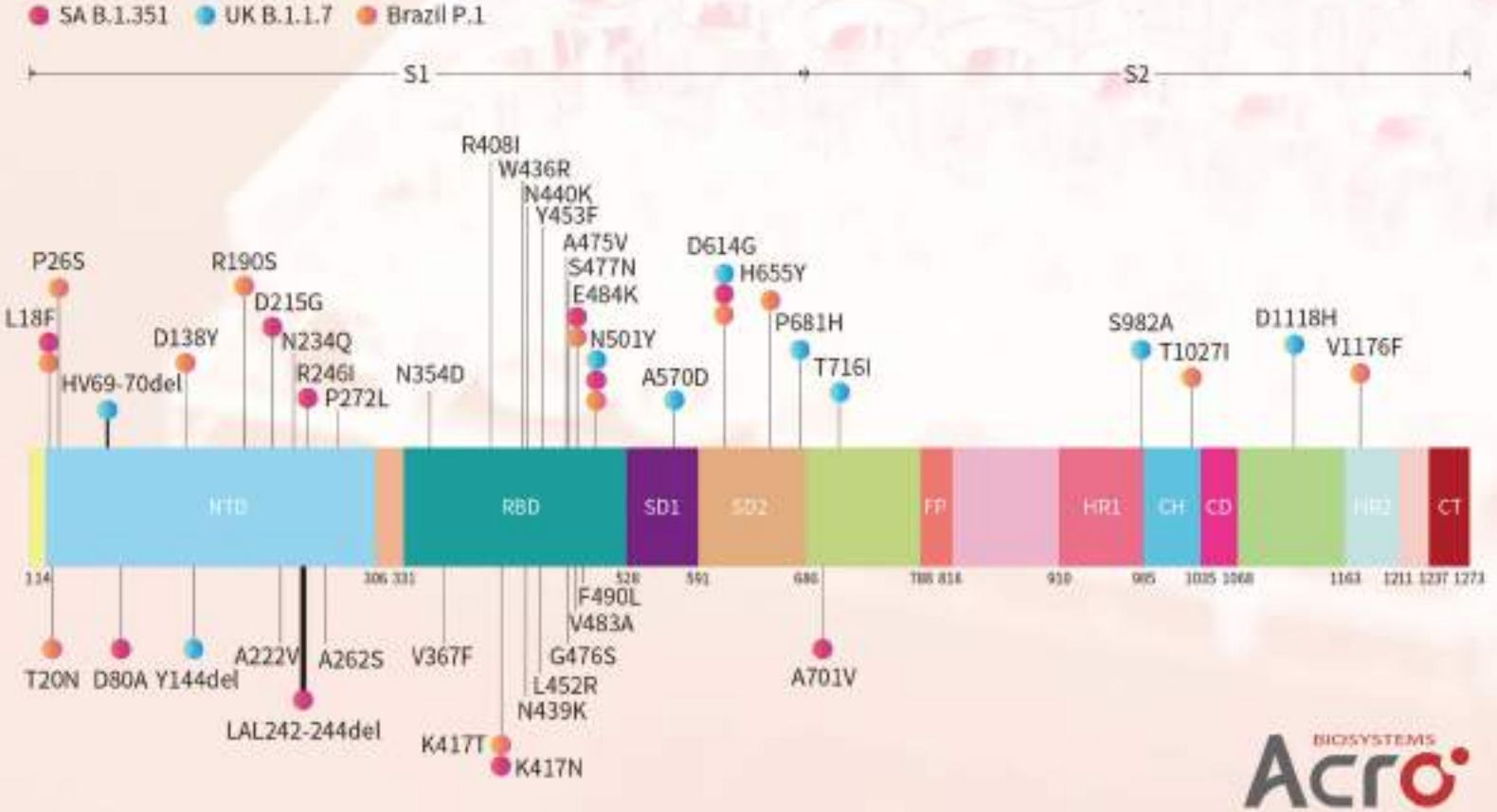
- K417N E484K N501Y D614G .....

Mimicking the authentic variant

- Full length S trimer with multiple mutations

Specially designed structure

- 6P mutations stabilize the protein in its pre-fusion conformation
- 2A mutations abolish the S1/S2 furin cleavage site
- Retaining natural property



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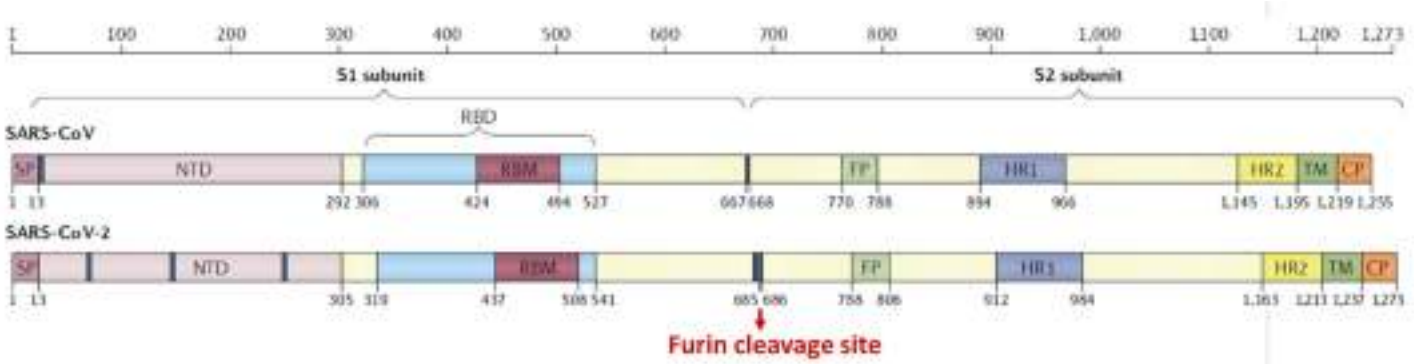


Figure 2. Structural schematic of S protein between SARS-CoV and SARS-CoV-2.[3]

S2 subunit comprises the fusion peptide, heptad repeats, transmembrane region, as well as cytoplasmic domain (Figure 2).

ACE2 is widely known as the key receptor for cellular entry of SARS-CoV-2. At the beginning of SARS-CoV-2 infection, ACE2 is like a "doorknob" allowing the virus to open the door to enter the cell.

In addition to ACE2, an interaction network of SARS-CoV-2 with host receptome through S protein showed that a number of host cellular receptors bind at least the two domains on S protein, RBD and NTD, suggesting both are critical for virus-host interaction.

Notably, the latest research indicated that the tyrosine-protein kinase receptor UFO (*AXL*) specifically might interact with the SARS-CoV-2 Spike protein and play a key role in promoting viral infection of the human respiratory system.[4]

A new frontier of emerging variants

Mutations in RBD have become a cause of concern as reports suggest higher transmissibility and reduced vaccine and antibody therapeutic efficacy in these strains.

As SARS-CoV-2 spreads globally, random mutations of the viral genome are inevitable. Mutations in and outside the RBD domain can significantly alter the conformation of S protein, which needs to be monitored continuously for effect in transmissibility and severity of COVID-19. GISAID is tracking new mutation/linages of SARS-CoV-2 in the course of this pandemic.

Nextstrain visualization of the SARS-CoV-2 mutation shows multiple clades of mutants emerging in 2019 and 2020 (Figure 3). However, mutations that lead to gain of functions are especially dangerous as it increases viral transmissibility and disease severity. Recently three new variants have emerged: 501Y.V1/B.1.1.7 from the UK, 501Y.V2/B.1.351 variant from S. Africa, and 501Y.V2/P.1 from Brazil. More recently, a U.S. variant originating on the west coast (B.1.429) has been identified and is suspected in a rapid increase of cases.[5]

The spread of these strains worldwide has been alarming due to the suspected increase in transmissibility and potential resistance to vaccine induced/therapeutic neutralizing antibodies. The mutation details for the variants are shown in Table 1.

Mutations in RBD have become a cause of concern as reports suggest higher transmissibility and reduced vaccine and antibody therapeutic efficacy

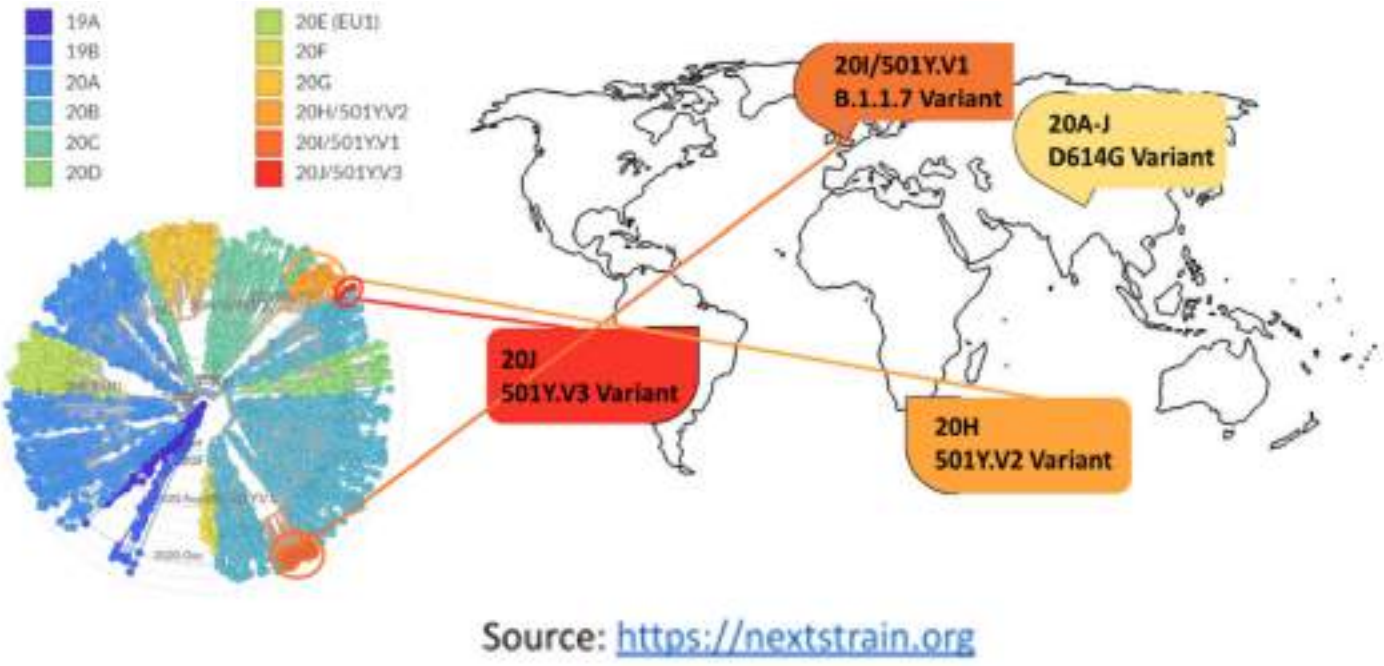
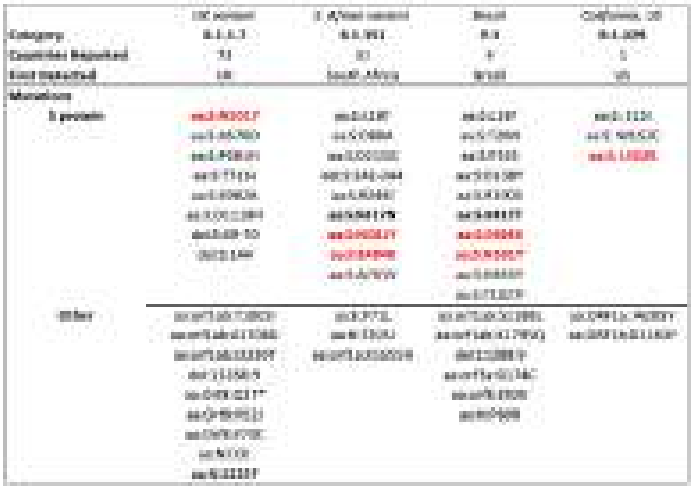


Figure 3. Phylogenetic clades of SARS-CoV-2 mutations with key variants around the world highlighted.

Table 1. Major SARS-CoV-2 variants in circulation worldwide.[3-5] Bold mutations are in the RBD and red colored mutations lie within the RBM.



in these strains. Recent reports suggest the B.1.1.7 variant is resistant to neutralization by antibodies targeting NTD of S protein and partially resistant to RBD mutants while showing modest resistance in convalescent plasma and vaccinated sera.[6] However, the B.1.351 has shown stronger resistance to both NTD and RBD antibodies and significant resistance (11–33 fold) in convalescent plasma and vaccine sera (6.5–8.6). With Pfizer/BioNTech, Moderna and Novavax vaccines, the antibody titer shows no difference against B.1.1.7 variant while lowered efficacy has been observed against B.1.351 variant.[6,7]

The E484K mutation present in both S. Africa and Brazil variants is thought to contribute to resistance where neutralization reduction greater than 10-fold was observed in some convalescent serum antibodies.[10] Another mutant, N501Y found in the UK, S African, and Brazil variant is thought to improve transmissibility of SARS-CoV-2 via strengthening ACE2 binding.[9]

In addition to effecting vaccination, these emerging variants have been found to lower potency of current antibody therapies against COVID-19. Two

Table 2. Vaccine efficacy analysis against emerging strains.[6-8]

Sponsors	Name	Type	Approval Date	Country/ Agency	Efficacy	Mutant Efficacy	Mutant Data
[illegible]	[illegible]	[illegible]	[illegible]	FDA	[illegible]	Yes	[illegible]
[illegible]	[illegible]	mRNA, S protein	[illegible]	FDA	[illegible]	No Partial	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible] [illegible]	Yes No	[illegible] [illegible]
[illegible]	[illegible]	[illegible]	Not Approved	N/A	[illegible]	No Partial	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	FDA	[illegible]	Partial Partial	[illegible]

Table 3. Neutralizing antibody therapy efficacy against emerging strains.[3,10]

Sponsors	Name	Type	Approval Date	Country/ Agency	Efficacy	Mutant Efficacy	Mutant Data
[illegible]	[illegible]	[illegible]	[illegible]	FDA	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	[illegible]	FDA	[illegible]	N/A	
[illegible]	[illegible]	[illegible]	[illegible]	FDA	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	[illegible]	FDA	[illegible]	[illegible]	

monoclonal antibody cocktails from Eli Lilly (bamlanivimab/estesevimab) and Regeneron (Casirivimab/imdevimab) that have received emergency use authorization from FDA have been tested against these variants. In the case of Eli Lilly's antibodies, bamlanivimab was susceptible to the E484K mutation in RBD leading to more than a 100-fold reduction in neutralizing activity while estesevimab retained activity with only a 4.4-fold decrease in neutralizing ability.[10] Regeneron's casirivimab and bamlanivimab both showed little to no effect in potency against the B.1.1.7 variant, while showing total or significantly abolished activity against B.1.347 variants.[6] Results are summarized in Table 3.

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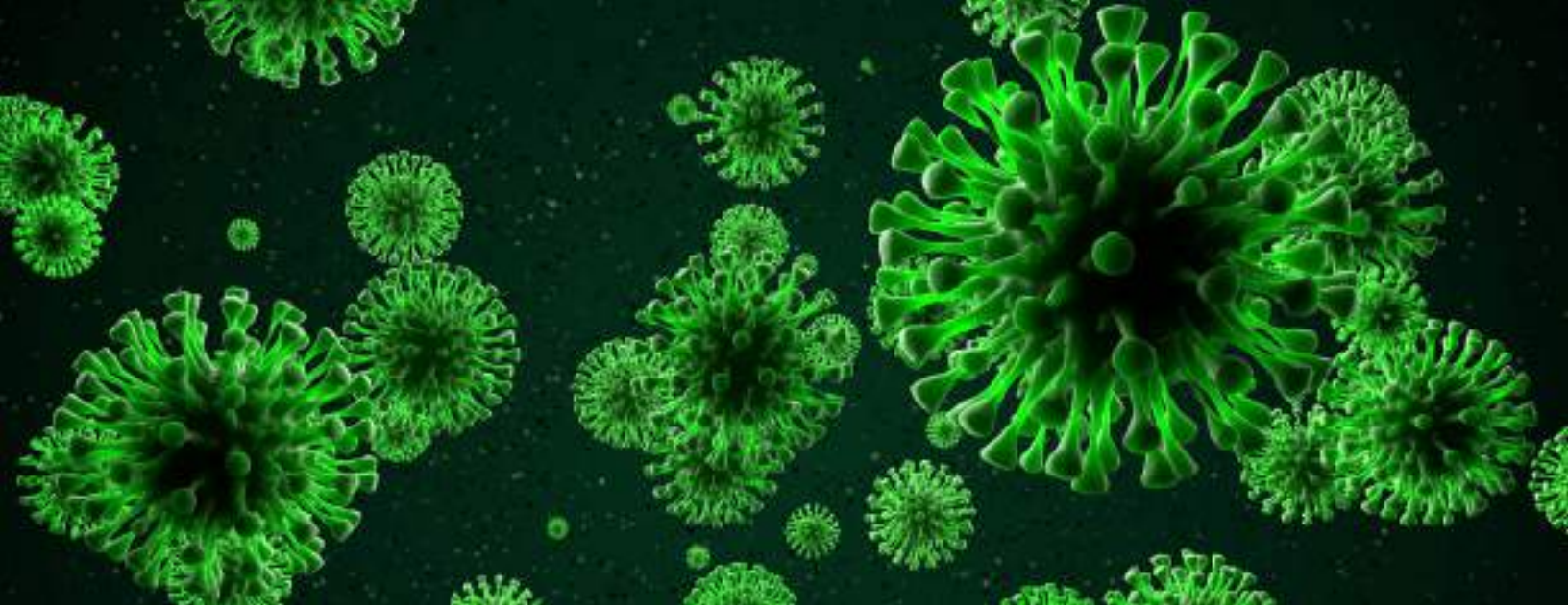

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Additional Resource

SARS-CoV-2 Related Products

ACROBiosystems has developed a comprehensive line of critical reagents, including recombinant proteins, antibodies, kits, beads, and so on, covering the critical targets of SARS-CoV-2. We hope that we can help to accelerate the research and development of SARS-CoV-2 related therapeutics, diagnostics, and vaccines with these high-quality products and advanced technical support.

Accelerating Discoveries for Viral Biology and Host Immunity with Advanced Cell Analysis Solutions

Optimizing the characterization of viral biology and the host immune response is essential to expedite drug development.

Jill Granger

Introduction

In the wake of global pandemics such as COVID-19, MERS-CoV, and Ebola, virology studies take center stage, capturing the attention of both scientists and the public alike. But within this "pathogen cloud" lies a silver lining—increased investment in viral biology research, the establishment of new scientific collaborations for rapid discovery, and renewed curiosity among the general public on the long-term health impact of emergent pathogens.

This increased awareness is accompanied by an urgent need to characterize viral biology and the host immune response for the identification of therapeutics and development of vaccines. Successfully executing a rapid, effective response to emergent pathogens requires the use of experimental approaches that are high throughput, yet properly balanced with thorough analysis for scientific rigor, leading to reproducible results. Traditional endpoint analysis can be limited, utilizing time-consuming, tedious "hands-on" workflows with multiple instruments, which also presents safety concerns through increased exposure to pathogens. The experimental manipulations of these assays introduce environmental fluctuations and lack of control that are not truly representative of host physiology. Traditional end-point assays lack kinetic insight on viral biology and immune responses, quickly consume limited sample and reagents, and require multiple instruments and software that could introduce variability. Taken together, traditional analysis assays are hampered by a slow time-to-result, lack kinetic information, and present workflow challenges that are problematic for fast-paced therapeutic screening and vaccine development. This is especially important during emerging infections when time-to-result is critical, but may impede other translational virology research activities, such as the development of therapeutics for regenerative medicine and cancer vaccines using oncolytic viruses.

The Incucyte® Live-Cell Analysis System and iQue® 3 Advanced High-Throughput Flow Cytometry platforms address many of these challenges, providing rapid, yet comprehensive and insightful analysis of vi-

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The COVID-19 pandemic has scientists developing novel life-saving treatments at breakneck speed. Sartorius provides innovative high-throughput & automated solutions to accelerate discovery, characterization & development.

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Simplifying Progress

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ral biology and host immune response studies. These instruments provide the automation, flexible applications, and throughput needed to quickly analyze the dynamics of viral infection and the immune response with reproducibility to provide new insights.

The Incucyte® incorporates automated image acquisition and analysis while maintaining physiological conditions in the user's incubator for better physiological control, quantifying dynamic changes in real time for hours, days, or weeks. The system's flexible applications, multiplexing capabilities, non-perturbing reagents, and integrated, user-friendly software permit rapid evaluation of cell health, function, movement, and morphology in a physiologically relevant environment while minimizing operator exposure to pathogens via remote access.

The iQue® 3 is an automated, high-throughput, advanced flow cytometry platform that allows users to rapidly multiplex protein analysis, immunophenotyping and perform functional assessments. Users can assess immune cell activation, perform functional profiling and antibody screening. This comes with the convenience of ready-to- use kits including the iQue® T Cell Phenotyping kit, Human Qbeads® Inflammation Panel for cytokine storm assessment, and kits for T cell activation, memory, killing, and exhaustion along with cytokine profiling for greater indepth characterization of the host immune response in each sample. Miniaturization is possible, as the platform requires only small sample volumes and is reagent sparing. It accommodates a variety of microplates, including 384-well for high-throughput analysis in minutes for profiling and pharmacological screening. Researchers can generate reproducible, high throughput tracking and analysis with integrated, user-friendly Forecyt® software. This timesaving, streamlined software offers plate-level annotation, analytics, and results visualization tools ideally suited for screening large data sets. Users may also access linear range data for deeper understanding on assay performance. Data from wells may be linked together and multiple assay outcomes combined to facilitate "hit" identification.

The research studies that follow provide real-world examples of how Incucyte® Live-Cell analysis and iQue® 3 Advanced High-Throughput Flow Cytometry were incorporated into virology workflows to provide rapid analysis solutions, simplification, and enhanced throughput, aiding the discovery of new insights on the host-pathogen life cycle throughout the course of infection. Additionally, post-infection, these platforms were used to help monitor exposure, assess immune protection, and accelerate the development of anti-viral small molecules, neutralizing antibodies, and vaccines.

Host Infection and Viral Replication

The host | pathogen lifecycle involves a series of dynamic events, including viral fusion, entry, replication, and spreading. Understanding the kinetic biology of these early events, including rates of infection and replication, can provide important clues for the identification of therapeutic targets, neutralizing antibodies, and vaccines.

Selecting and Developing a Viral Infection Model

A critical, first step for virology studies is the selection of an appropriate model that is both biologically and clinically relevant. Roberts *et al* (2017) evaluated cell lines to identify the most physiologically relevant as an *in vivo* model for the infectious cycle of Chikungunya virus (CHIKV).[1] Incucyte® Live-Cell Imaging and Analysis was used to capture Brightfield images of cell morphology during differentiation, better recapitulating *in vivo* conditions within the physiologically relevant environment of the incubator. Morphological changes during C2C12 (myoblast) and Huh7 (hepatocyte) differentiation were visualized. C2C12 cells were serum-starved

in vitro, differentiated, and formed myotubes, similar to *in vitro* muscle tissue. This was also associated with enhanced CHIKV RNA replication. Huh7 cells differentiated with DMSO underwent arrested growth and up-regulation of liver proteins, showing reduced levels of CHIKV RNA replication compared to that of undifferentiated cells. This illustrated how important morphological information from live-cell analysis can add to biological understanding.

The development of new infection models may be required to enable the safe handling of viruses. There is limited availability of BSL-4 facilitates that can safely accommodate particularly virulent viruses, which may impede research progress. Xiao *et al* (2018) developed an Ebola virus pseudotype (E-S-FLU), a single-cycle virus capable of mimicking Ebola cell entry, which could be safely handled in BSL1/2 containment facilities.[2] The E-S-FLU virus was created using an influenza virus that had been coated the glycoprotein from Ebolavirus (EBOV-GP). E-S-Flu encoded a green fluorescent reporter that replaced the hemagglutinin (HA) gene from influenza. A lentiviral vector was used to transduce MDCK-SIAT1 cells that stably expressed high levels of EBOV-GP (E-SIAT). As part of the E-S-Flu viral titer characterization, the Incucyte® was used to obtain images of plaques at 3 h intervals, revealing key morphological clues during infection kinetics: E-SIAT cells formed small diffuse plaques, while H5-SIAT cells produced denser plaques. A single production batch of the pseudotyped, E-S-FLU virus was leveraged to screen a library of 1280 compounds as BSL1/2 for inhibition of viral entry.

Viral Entry and Infection

Characterizing viral entry and infection can provide information that may be exploited for therapeutic target identification or vaccine development. Stein *et al* (2019) examined Human cytomegalovirus (CMV) entry into host cells for therapeutic target identification using a library of monoclonal antibodies directed against human retinal pigmented epithelial cell surface proteins (ARPE).3 Mice were injected with an ARPE-19-derived membrane fraction to produce immunized sera, which was analyzed for antibodies against ARPE-19 cell surface proteins using iQue® Advanced High-Throughput Flow Cytometry. A viral inhibition assay identified two clones that caused inhibition yet failed to block entry into fibroblasts. Subsequent experimentation led the authors to propose that CD46 viral entry is dependent on a post-binding event, wherein CD46 interacts with CMV PC. Studies using CD-46 KO epithelial cells showed a reduction in CMV dissemination, indicating that a CD46-dependent process may be involved in viral spreading. The authors further related the advantages of using this mAB library approach over genetic screening, including the lack of manipulation of transcriptional profiles, elimination of transfection and transduction toxic effects, as well as flexibility for assay customization.

Viral infection and entry can also be studied using live-cell imaging and analysis to monitor signals from fluorescent reporters in treated cells. Qui *et al* (2018) studied the interactions between the Ebola virus (EBOV) glycoprotein with Niemann-Pick C1 (NCP1) to gain insight on trafficking and delivery of EBOV to NCP1.[4] Incucyte® Live-cell Imaging and Analysis was used to capture the entry of GFP- expressing MLV pseudotypes harboring EBOV ΔM GP or VSV G in ArPIKfyve and Sac3 KO cells through the visualization of GFP+ foci. Additional analysis showed that all subunits of the PAS complex were required for EBOV entry, and that cellular production of PtdIns (3, 5) P is needed to promote efficient delivery to NPC1. Taken together, these results shed new light on the infection and entry of this virulent pathogen.

Viral Replication

In addition to viral entry, viral replication represents another area where these platforms may provide new insights for therapeutic exploitation. Rothan

et al (2019) studied the use of host cell components as an alternative approach for targeting viral pathogens in dengue virus (DENV) and Zika virus (ZIKV) infections.[5] Live-cell analysis was used to quantitate real-time proliferation of ZIKA-infected and DENV-infected Vero cells treated with Bardoxolne methyl (CDDO-me) or neutralization with ZIKV-neutralizing antiserum. CDDO-me is an inhibitor of the Hrd1 ubiquitin ligase-mediated ERAD pathway that possibly targets host factor grp94 and was found to inhibit dislocation. ZIKV- infected Vero cells were treated with CDDO-me or ZIKV-neutralizing serum, and the Incucyte® was used to automatically generate kinetic processing metrics from the imaging. The percentage confluence over time was calculated to determine the cytopathic effects of infection.

CCDO-me (0.13 µM) protected the Zika-infected Vero cells as well as 5% ZIKV-neutralizing antiserum. CDDO-me and PU-WS13 (a grp94 inhibitor) also had anti-flaviviruses activity, and inhibition of grp94 may play a role in CCCO-me inhibition of both DENV and ZIKV. Herod *et al* (2019) used live-cell imaging and analysis to detect GFP reporter gene expression to study the effects of the anti-influenza drug Arbidol (ARB) against the foot-and-mouth disease (FMDV) and equine rhinitis virus (ERAV).[6] Using replicons in a FMDV replication assay, they were able to study viral replication at the BSL2 level of containment. BHK-21 cells were treated with DMSO, GuHCl, or ARB and transfected with a wild-type GFP FMDV replicon. Expression of the fluorescent reporter protein was monitored for 10 h using the Incucyte®, revealing that ARB treatment produced dose-dependent suppression of FMDV replication. Subsequent analysis showed that ARB inhibited the replication of FMDV RNA sub-genomic replicons, suggesting a possible inhibition of picornavirus genome replication.

Viral Transmission

In addition to viral replication, live-cell analysis can provide new insights into viral transmission and spread. In the case of Measles Virus (MeV), the virus can form syncytia when uninfected cells fuse with infected cells expressing F- and H- glycoproteins from the viral envelope. MeV, part of the *Morbilliviruses*, may also spread by viral budding. Kelly *et al* (2019) used live-cell analysis to study the cell-to-cell spread of Measles Virus (MeV) using a recombinant MeV that expressed a GFP fluorescent protein as a transcription unit.[7] The Incucyte® was used to quantitate viral replication, syncytia formation, and expansion, revealing that a protein induced by interferon, bone marrow stromal antigen 2 (BST2), inhibited the replication of MEV-GFP. These findings were also concordant with a reduction in MeV nucelocapsid (N), F and H proteins as detected by western blot. Additional analysis with Incucyte®, immunolabeling, and biochemistry were utilized to determine that BST2 inhibited cell fusion (visualized as reduced syncytia formation) and cell-cell spread.

Another important point of study for viral transmission is the element of viral dispersal. Viruses may be dispersed through multi-viron assemblies that infect the host cells. Anderu-Moreno and Sajuan (2018) studied the viral fitness of free vesicular stomatitis virus (VSV) particles verses those that were aggregated in saliva.[8] This study leveraged the kinetic capabilities of real-time, live-cell imaging and analysis with Incucyte® to compare fitness effects of viral particles dispersed in aggregated form to those in monodisperse. Real-time, live cell kinetic studies were used to study mouse embryonic fibroblasts (MEFs) inoculated with either monodispersed VSV-mCherry particles or aggregated VSV-GFP using human saliva.

The growth curves, generated from the Incucyte® analysis, revealed leftward shift for viral populations formed by aggregates, as compared to those which were monodispersed. Aggregation of virus particles imparted a short-term fitness advantage and increased the release of viral progeny, which was dependent on cellular permissively to infection and was correlated with the level of cellular innate immunity.

Host Immune Responses

Viral biology doesn't occur in isolation but is subject to the forces of the host immune response, which is delicate interplay between the innate and adaptive immune systems that ebbs and flows as the pathogen is detected, combatted, and eliminated. The dynamics and extent of this response is complicated by individual genetics and inflammatory status, as well as viral mutational shifts. Kinetic, comprehensive analysis may uncover key information on the timing and intensity of this dynamic, often complex story. Changes in cellular health, function, phenotype, and activity, as well as cyclic production of pro- and anti-inflammatory mediators throughout the course of infection can hold critical information about how the virus | immune system interactions and response to therapeutic candidates.

Immune Cell Activation

Viral exposure initiates immune cell activation and sustained responses for pathogen elimination. Zander *et al* (2019) studied how CD4+ T cells can help sustain exhausted CD8+ T cells during chronic infection and cancer.[9] The authors performed a functional assessment of CD8+ T cells that were specific for the GP33-41 peptide of lymphocytic choriomeningitis virus (LCMV). Subsets of sorted GP33-41 CD8+T cells were co-cultured with target EL4 ells treated with GP22 peptide or a control peptide. The authors used Incucyte® S3 and Incucyte® Annexin V Red Reagent to quantitate cytotoxicity of the CD8+ T cells. Using additional scRNA-seq analysis and transcriptional profiling, they discovered that the CX3CR1+-expressing CD8+ T cell subset had a cytolytic function that was key in viral control. This was also dependent on IL-21 produced from the CD4+ T cells.

In another CD4+ T cell study, Kwon *et al* (2020) examined latent HIV-1 proviruses in human resting memory CD4+ T cell subsets to better understand latency and escape of HIV-1 from immune clearance.[10] A multiple stimulation viral outgrowth assay was used to test naïve and memory T cells (central, transitional, and effector) from HIV-1 patients on antiretroviral therapy. The iQue® Screener Plus was used to assess cellular activation levels of resting CD+4 T cell subsets, finding no correlation between the subsets and rounds of stimulation needed to produce viral outgrowth. Additional analysis revealed a lack of enrichment for intact or inducible proviruses in the T cell subsets, highlighting the complexity of pursuing T cell memory subsets for HIV-1 therapy.

Antibody Responses and Epitope Mapping

In addition to activation of innate immune responses, the adaptive immune system is called into action to produce antibodies for viral defense. Epitope mapping of viral antigens is an important characterization for study of early responses, and advanced high-throughput flow cytometry is ideally suited to supplement such investigations. In an insightful study by Williamson *et al* (2019), the authors analyzed early convalescent memory B cells from survivors of Ebola infection.[11] Monoclonal antibodies (mAbs) from the memory B cells were isolated from survivors 1–3 months post infection to examine the frequency of Ebola- specific B cells. The iQue® Advanced Flow Cytometry Platform was used as part of the alanine-scanning shotgun mutagenesis for epitope mapping. An alanine scanning mutagenesis library was created for Ebola glycoprotein (EBOV GP) to generate alanine-scanning mutagenesis library clones. HEK-293T cells were transfected with the library, mutant GPs expressed, and the cells fixed and stained for flow cytometry with a Fab fragment of EBVOV237 and a secondary Alexa Fluor 488 conjugated- antibody. The iQue® was used for analysis of cellular fluorescence. The reactivity of EBOV237 Fab fragments to the mutants was compared to the wild-type EBOV GPΔmucin. Clones that did not bind the EBOV237 Fab fragment, but bound to other control

EBOV mAbs, were deemed clones of interest. Subsequent analysis identified a neutralizing epitope in the glycan cap of the surface glycoprotein, recognized by a mAB designated as EBOV237. This epitope may play an important role in the early response to Ebola virus and be a potential vaccine candidate.

In another study using alanine mutagenesis, Collins *et al* (2019) studied the human B cell antibody response to Zika virus infection.[12] Two type-specific mAbs were isolated from a SIKV patient with protective antibodies and were used to map viral epitopes. Alanine scanning mutagenesis was carried out to generate a ZIKV prM | Eslanin-scan library with mapping of cells expressing ZIKV E mutants. The throughput of the iQue® Advanced Flow Cytometry Platform enabled rapid assessment of mean cellular fluorescence for the epitope mapping. The authors concluded that both the plasma antibody and memory B cell responses are ZIKV-type specific. The neutralizing antibodies produced primarily target quaternary structure epitopes on the viral envelope, which the authors noted had been found in DENV and other flaviviruses. They emphasized that the identification of targets that produce a long-lived neutralizing Ab response was informative, and may be used to guide antigen design and immunity generated by vaccines.

Cell Death

Understanding the kinetics of cell death mechanisms can provide some valuable, and even previously unknown, insights for understanding immune cell killing and cell death mechanisms of virally infected cells and identify new therapeutic targets.

One area of cell death that has received increased attention in viral disease is NETosis. In a ground-breaking study by Barr and Roderiguez-Garcia *et al*, the authors identified NETosis formation by neutrophils in genital mucosa as a protective defense against HIV infection, a previously undescribed finding in the early stage of HIV infection.[13] Live-cell imaging and analysis with Incucyte® captured the *in vitro* dynamics of GFP-tagged HIV-VLP (HIV viral-like particles) entrapped by human genital neutrophils (Figure 1). NETs were released within minutes of viral exposure.

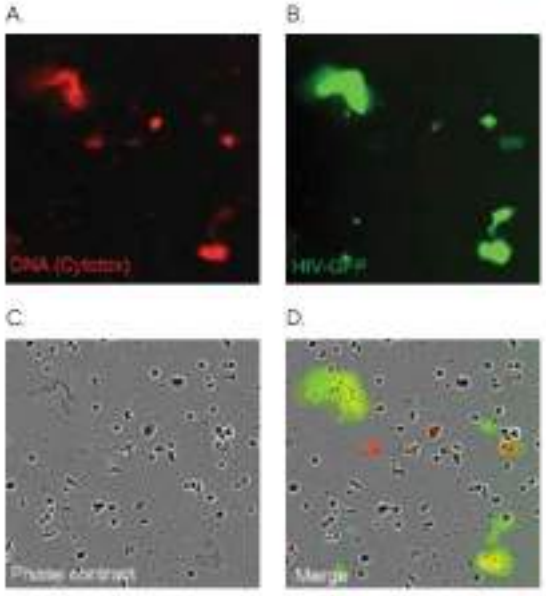

Figure 1. Neutrophils isolated from the female genital tract cultured in impermeant DNA dye (red) and stimulated with GFP-labeled HIV viral-like particles (green) to induce NET formation. (A) shows the DNA staining in NETs (red). (B) shows the GFP-labeled viral-like particles entrapped in NETs. Bottom left panel (C) shows the phase contrast, in which NETs can also be visualized, and bottom right panels (D) shows the merge with HIV trapped in the NETs appearing as yellow. Image courtesy of Dr. Marta Rodriquez-Garcia, Tufts University School of Medicine.

In another NETosis study, Drs. Chirivi, Raats, and colleagues (at ModiQuest B.V.) reported on NET release into the extracellular environment during inflammation.[14] Incucyte® Live-Cell Imaging and Analysis was used to capture NET release from neutrophils in the presence of an engineered therapeutic anti-citrullinated protein antibody (tACPA), which showed both therapeutic and prophylactic potential for inflammatory disease associated with NET pathology, being the first in kind described to interfere with NET expulsion into the extracellular space.

In addition to NETosis, cell death may also arise from apoptosis. Brown *et al* (2018) examined the interaction of critical proteins involved in viral infections of poultry: the Meq from Marek's disease virus (MDV) and the apoptin protein in chicken anemia virus (CAV).[15] Incucyte® Live-Cell Imaging and Analysis and Incucyte® Caspase-3/7 Apoptosis Reagent were used to study the functional, kinetic effects of Meq-apoptin interactions on apoptosis on transfected chicken fibroblast cells (DF-1). Meq protein inhibited apoptin, which may have important implications for CAV vaccine production. Cubas-Gaona *et al* (2018) examined the loss of IgM B lymphocytes in Infectious Bursal Disease (IBDV) and the role of type 1 interferon (IFN) in IBDV infection.[16] Live-cell analysis was used to examine enhanced apoptosis of HeLa cells treated with IFN-α and infected with IBDV and DF-1 cells. This study demonstrated that IBDV-infected cells undergo marked apoptosis when exposed to IFN-α.

A third mechanism of cell death is necroptosis, and viruses may avoid destruction by exploiting this mechanism to promote infection. Reddy *et al* (2020) studied the role of Nr-13, a Bcl-2 related protein, in the death of avian B cells.[17] Herpesvirus of Turkey (HVT) VNR-13 is an alpha herpesvirus-encoded Bcl-2 homolog, and a lab-generated mutant (HVT-ΔvNr-13) was used to study the functional role of VNr-13. Live-cell analysis was used to monitor apoptosis of DF-1 cells infected with HVT vNr-13 transfected cells, showing that HVT vNr-13 is involved in the inhibition of cellular apoptosis at later stages of viral replication along with an increase of PFU. HVT was found to block apoptosis in cells that were infected, but also to activate apoptosis in bystander cells that were not infected.

Chemotaxis

In addition to cell death, chemotaxis may be upregulated during infection. Live-cell analysis is ideally suited for the high-throughput kinetic measurement of chemotaxis studies for drug discovery screening. Chen *et al* (2018) used live-cell chemotaxis analysis of immune cells for therapeutic target identification, mechanism of action studies and safety assessment.[18] Incucyte® Chemotaxis Assays enable users to automatically monitor and quantitate cell migration horizontally, prior to vertical movement, through the pores of biologically relevant surfaces at high throughput. This comes with the added benefit of also capturing morphological changes during this process. Salyer and David (2017) used this chemotaxis assay to aid in the development of a safe and effective vaccine adjuvant.[19] They examined structure-activity relationships focused on TLR-containing adjuvants associated with inflammatory and myeloid-associated modules, identifying transcriptomal signatures of innate immune stimulating molecules for use as suitable vaccine adjuvants. Incucyte® Live-Cell Imaging and Analysis was used to assess PBMC chemotaxis with HFFs (human foreskin fibroblasts) treated with graded concentrations of TLR agonists. The HFFs stimulated with TKR2/6 agonists displayed functional chemotactic responses from lymphocytes in the PBMCs, while TlR1/2 did not.

Cytokines, Chemokines, and Cytokine Storms

As mentioned above, the production of cytokine and chemokine proteins can be key indicators on the host immune responses against viral infection. Understanding the biology of these important effector proteins, the cells that produce them, the kinetics of their release, and functional effects can shed new light on immune system activities and the underlying pathology of viral infections.

As a case in point, the biology of the pro-inflammatory cytokine MIF (Macrophage Inhibitory Factor) has an interesting story when it comes to the immune response to disease and viral infection. MIF can regulate the innate immune response through a variety

of means, including inhibition of apoptosis, activation of macrophages, and stimulation of NLRP3 inflammasome. Karsten (2017) undertook an investigational doctoral study to characterize the role of red blood cells in inflammatory cytokine signaling.[20] Incucyte® Live-Cell Analysis was used to study the proliferation and confluence of cell from the A549 cell line incubated with red blood cells, which was also accompanied by release of cytokines in lysates from the red blood cells. The primed RBCs were able to stimulate T cell proliferation and activation. Karsten found that red bloods cell served as a reservoir for not only MIF, but also other effector proteins, supporting the concept that red blood cells acted as a buffer, binding and releasing cytokines to affect other immune cell activities. Smith *et al* (2019) later examined the role of MIF in a mouse model of influenza (IAV, strain PR8) to understand macrophage regulation during viral infection. MIF-deficient mice, infected with influenza, had less inflammation, reduced viral load, and lower mortality as compared to WT controls.[21] MIF increased inflammation in the airspaces of the lung and increased the lung's permeability during infection.

Mice overexpressing MIF in alveolar epithelia had more inflammation, viral load, and higher mortality. Cytotoxicity assays and apoptosis assays using Incucyte® Live-Cell Imaging and Analysis, as well as the Incucyte® apoptosis reagents Caspase-3/7 and Annexin-V, were used to assess IAV viral replication in GFP-labeled bronchoalveolar lavage (BAL) cells from IAV infected mice and cell viability (Figure 2). This analysis helped to reveal that MIF promoted viral spread. Human lung epithelial cultures treated *in vitro* with recombinant human MIF displayed an increased number of influenza virus-infected cells. The authors concluded that MIF impaired the antiviral immune response of the host in the tested models and augmented the inflammatory process during influenza infection. Such findings prompt further questions on the biology of this important cytokine,

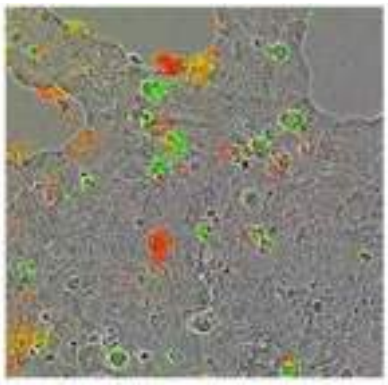

Figure 2. Calu-3-lun carcinoma cells infected with Influenza A (PR8), treated with human recombinant Macrophage Migration Inhibitory Factor (MIF). Incucyte® Annexin V used for viability assessment. Image courtesy of Dr. Candice Smith.

its associated pathology, and the possible effects of inappropriate upregulation that might occur in susceptible individuals.

Although an immune effector response can be beneficial, the dysregulation of immune balance between pro- and anti- inflammatory activities can prove disastrous for the host. Nowhere is this more apparent than in the development of cytokine storms and acute respiratory distress syndrome (ARDS) in response to viral and bacterial infections. Hillyer *et al* (2017) explored the balance of IFN (interferon) and proinflammatory cytokines during respiratory syncytial virus (RSV) infection and how this might restrict viral replication and impact disease severity.[22] Incucyte® analysis was used to assess viral spreading in rgRSV- infected A549 (alveolar) and REAS-2B (bronchial) cells along with videos of viral infection. Cytokines were assessed in cell supernatants along with western blots of transcription factors and pattern recognition receptors. The A549 cells were found to be highly permissive of infection and expressed genes characteristic of a pro- inflammatory response. Further, BEAS-2B cells infected cells had small foci with greater expression of antiviral interferon-stimulated genes (type I and II) and pattern

recognition receptors. These insights emphasized the role of the established antiviral state of cells and how they respond differently to infection.

Post-Infection Analysis and Protective Immunity

Post-infection studies are a critical component in the assessing of the extent of infection and possible sustained immunity. This is true of both pathogenic viruses, and those designed for therapeutic purposes. Serology studies on antibody titer, immune cell memory and exhaustion, and effects on host tissue, can provide important information on the long-term effects of infection on the host.

Serology studies post-infection can provide a wealth of information on protective antibodies. Xu *et al* (2016) studied immune protection from anamnestic antibody during acute Dengue virus infection.[23] Antibodies, cloned from patient samples with heterologous secondary infection of dengue virus, were evaluated for their anamnestic response following symptomatic reinfection with a heterologous serotype of DENV. The dengue antibodies were produced by human plasmablasts shortly after a secondary infection. The iQue® Advanced High-Throughput Flow Cytometry Platform was used during shotgun mutagenesis epitope mapping to test antibody reactivity against mutant clones. Despite neutralization *in vitro* and a reduced viremia in an *in vivo* mouse model, the cross-reactive anamnestic antibody response was protective. Niu *et al* (2019) examined the antibody responses of convalescent patients infected with Zika virus (ZIKV), isolating three mAbs which bound to ZIKV envelope protein that were most potent in neutralizing the virus.[24] The iQue® was used to perform epitope mapping of the antibodies. A lethal dose of ZIKV was administered to SCID mice, and the antibodies provided protection.

Stewart *et al* (2014) described the use of Incucyte® analysis to determine Hepatitis C virus (HCV) titer in infected Huh-7 cells.[25] Infected cells were counted, and software was utilized to extrapolate titer without expression of a reporter gene, which was indicated as infectious units per milliliter (IU/mL). Using this method, the authors noted a reduction in cost, time and error, compared to other means, with improved accuracy, precision, and reproducibility. Additionally, there was also a wider linear range of detection, making it highly suitable for high-throughput antiviral compound screening.

In addition to antibody titer, live-cell analysis can be used to assess proliferation and wound repair following viral infection, which may be related to pre-existing host physiology. Andersson *et al* (2020) studied impaired airway epithelial cell wound healing following infection with RSV in children with severe therapy resistant asthma (TRA) and preschool-aged children with wheezing (PSW).[26] Incucyte® analysis was used to assess airway epithelial cell proliferation and scratch wound repair following exposure of PBECs (primary bronchial epithelial cells and biopsies) isolated from severe PSW and STRA patients to house dust mite allergen (HDM) and the TLR3 agonist ds RNA poly I:C and IL-33. The PSW children demonstrated impaired airway epithelial proliferative responses and wound healing, demonstrating the influence of a reactive airway on the host response to viral infection.

Development of Therapeutics and Vaccines

In the early stages of emerging viral infections, the search for therapeutics may focus on the repurposing of existing drugs, such the early COVID-19 investigative use of chloroquine (CQ) and hydroxychloroquine (HCQ). In bioRxiv pre-print, Yang *et al* (2020) reported on the effects of chloroquine (CQ) and hy-

droxychloroquine (HCQ) in cell lines, examining cytotoxicity and assessment of physiologically based pharmacokinetic models (PBPK) for risk prediction. Incucyte® Live -Cell Analysis was used to monitor cell viability and proliferation in the cytotoxicity assessment of CQ and HCQ.[27] The authors noted that cytotoxicity was both time- and dose-dependent *in vitro*, which suggested a short period of administration may be appropriate if delivered clinically.

Elements of existing vaccines may also be modified as a development strategy. Behzadi *et al* (2019) repurposed the influenza A vaccine platform via reverse genetic engineering to express a neutralizing antibody against the CMV viral envelope protein (gH).[28] Here, advanced high-throughput flow cytometry was used to perform binding analysis of viral envelope protein for the design of a new human cytomegalovirus (CMV) vaccine. Boudreau *et al* (2020) used a systems serology approach to assess antibody functional profiles of individuals given an H5N1 avian influenza vaccine administered with the adjuvants alum and MF59, or delivered unadjuvanted to examine the effect of MF59 on the generated immune response.[29] Antigen-specific antibody subclass | isotypes and FcR binding were determined using a high-throughput, bead-based assay that were read on an iQue® advanced flow cytometry platform.

Incucyte® Live-Cell Imaging and Analysis can also be used to verify that a vaccine candidate binds to a target. In the video to the right, Humane Genomics used this technology to examine the binding of a vaccine candidate to ACE2 receptors on target cells. In the video, the ACE2 receptor (red) on the cells is shown binding with the SARS-CoV-2 spike protein (green) on the vaccine. This binding is visualized as yellow, co-localized patches.

In another study of SARS-CoV-2 and SARS-CoV, Stukalov *et al* (2020) in the lab of Dr. Andreas Pichlmair, the authors used a multi-level proteomics approach to examine host-perturbation by SARS-CoV-2 and SARS- CoV.[30] As part of this assessment described in a bioRxiv pre-print, they used live-cell imaging and analysis to validate SARS-CoV-2 vulnerabilities that could be targeted for antiviral therapy using existing drugs. The authors discovered that SARS-CoV-2 perturbs a variety of host-pathways at multiple levels. Incucyte® Live-Cell Imaging and Analysis was used as part of a viral inhibition assay study, assessing cell viability and virus growth of A549-ACE2 cells treated with inhibitors and infected with SARS-CoV-2-GFP, as part of a time-lapse fluorescent microscopy assessment of SARS-C0V-2 GFP-reporter virus infection. This drug screen assessed 48 drugs that modulated pathways that were perturbed by SARS-CoV-2 to identify antiviral therapy candidates. Evaluation of GFP signal and cell growth from 48 h of live-cell imaging and analysis with Incucyte® were used to generate heatmaps of cell growth rate over time.

The compounds Gilteritinib, Ipatasertib, Prinomastat and Marimastat were found to have the highest antiviral effect and inhibited replication of SARS-CoV-2, yet displayed little effect on cell growth.

In addition to the development of vaccines for emerging pathogens, live-cell analysis may be useful for the development of personalized cancer vaccines. Sahin, *et al* (2017) described the development of individualized mutanome vaccines using RNA-based poly-epitopes for a first-in-human treatment for melanoma.[31] A comprehensive identification of each individual patient's mutations was performed along with computational prediction of the neo-epitopes. Customized vaccines were prepared based on individual mutational status. The Incucyte® system and Incucyte® Caspase-3/7 Green Apoptosis Reagent were used to assess the apoptosis of melanoma cells co-cultured with effector T cells *in vitro*, assisting with vaccine characterization. Hassanzadeh *et al* (2019) used an interferon sensitive mutant Maraba virus (MG1), to

better understand how oncolytic viruses interact with the host immune system and kill tumors.[32]

Incucyte® Live-Cell Imaging and Analysis was used to monitor the rate of viral propagation through confluency of GFP signal in WT and S51 mouse embryonic fibroblasts (MEFs). Incucyte® Cytotox Red Reagent was used to monitor the infection of mock- or infected cells over the time course of infection. This platform was also used to observe the effect of knocking down the translation factor eIF5B on cell viability and rate of infection with MG1.

Conclusion

These studies demonstrate the value of using the Incucyte® Live-Cell Imaging and Analysis platform and iQue® Advanced High-Throughput Flow Cytometry for virology research to provide new insights on viral biology and the host immune responses to infection. More examples can be found in the "Additional Reading" section below. The high-throughput capacity, flexible applications, analysis software, reagents, and kits enable users to quickly and comprehensively maximize the data collected from each sample in minimal time. Further, these platforms are both sample and reagent sparing.

Important kinetic events during the viral life cycle, and their impact on host cell health, function, phenotype, activity, and immune cell responses can be automatically assessed using small sample sizes to quickly generate reproducible, consistent experimental results.

Use of these platforms allows investigators to strike the ever-important balance between the "need for speed" during rapidly evolving public health issues and pre-clinical testing versus the thorough, quantitative, analysis of viral biology and immune responses with reproducibility and confidence. By simplifying analysis and increasing throughput, these tools can accelerate basic research and discovery throughout the host-pathogen life cycle. Post- infection, these assays can be used to monitor exposure, and rapidly characterize serological responses to assess protection or studies of viral latency. They can also be used for screening of anti-viral small molecules and neutralizing antibodies, and the identification and development of new vaccine candidates. Incorporating Incucyte® and/or iQue® platforms into experimental analysis can reveal some long- sought "missing pieces" in the complex biological puzzle of host-pathogen life cycle, and as the rapid emergence and persistence of viral infections have taught us, every piece may be needed.

Click here to view **References.**

About the author

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Additional Resource

Evaluating Viral Infections Across the Entire Host Pathogen Life Cycle - Infographic

Utlilizing Incucyte® Live-Cell Imaging and Analysis to Measure Viral Replication Overtime

IncuCyte™ Chemotaxis Cell Migration - Neutrophils

Evaluating Non-Pathological Virus Infectivity of Cells that Express ACE-2 with Live-Cell Analysis