



HHS Public Access

Author manuscript

J Proteome Res. Author manuscript; available in PMC 2023 December 13.

Published in final edited form as:

J Proteome Res. 2021 February 05; 20(2): 1133–1152. doi:10.1021/acs.jproteome.0c00764.

Proteomic approaches to study SARS-CoV-2 biology and COVID-19 pathology

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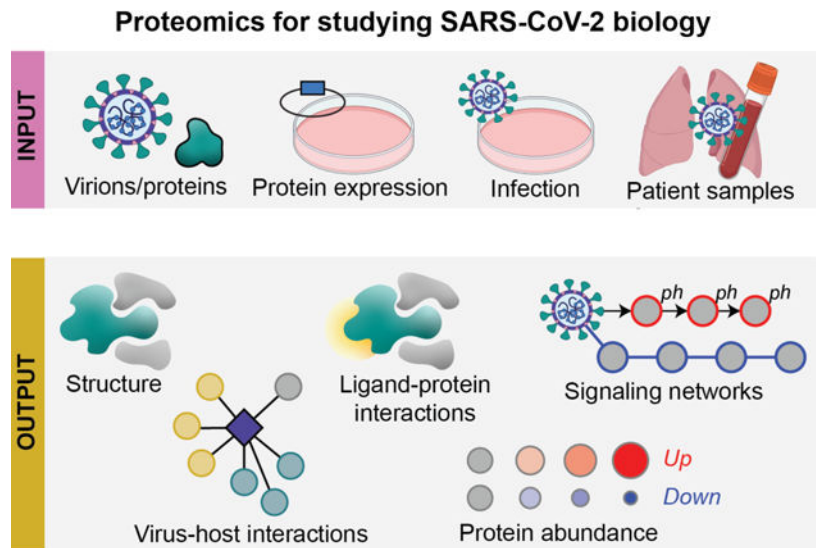
Abstract

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of Coronavirus Disease 2019 (COVID-19), was declared a pandemic infection in March 2020. As of December 2020, two COVID-19 vaccines have been authorized for emergency use by the U.S. Food and Drug Administration, but there are no effective drugs to treat COVID-19 and pandemic mitigation efforts like physical distancing have had acute social and economic consequences. In this perspective, we discuss how the proteomic research community can leverage technologies and expertise to address the pandemic by investigating four key areas of study in SARS-CoV-2 biology. Specifically, we discuss how (1) mass spectrometry-based structural techniques can overcome limitations and complement traditional structural approaches to inform the dynamic structure of SARS-CoV-2 proteins, complexes, and virions; (2) viral-host protein-protein interaction mapping can identify the cellular machinery required for SARS-CoV-2 replication; (3) global protein abundance and post-translational modification profiling can characterize signaling pathways that are rewired during infection; and (4) proteomic technologies can aid in biomarker identification, diagnostics, and drug development in order to monitor COVID-19 pathology and investigate treatment strategies. Systems-level high-throughput capabilities of proteomic technologies can yield important insights into SARS-CoV-2 biology that are urgently needed during the pandemic, and more broadly, can inform coronavirus virology and host biology.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Graphical Abstract



Keywords

SARS-CoV-2; COVID-19; proteomics; mass spectrometry; virus-host interactions; structural proteomics; protein-protein interactions; host response; drug discovery; biomarker discovery

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel coronavirus that causes Coronavirus Disease 2019 (COVID-19), and its outbreak in 2019 and subsequent pandemic devastated global economies and human health. The coronaviridae family of viruses, named for their crown-like appearance under an electron microscope, includes alpha, beta, gamma, and delta subgroups that infect a wide variety of mammals and birds, but mutations facilitate cross-species infections and human-to-human transmission of the viruses¹. Seven coronaviruses are known to infect humans, two alpha coronaviruses (HCoV-229E and HCoV-NL63), and five beta coronaviruses (HCoV-OC43, HCoV-HKU1, MERS-CoV, SARS-CoV-1, and SARS-CoV-2)². HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1 regularly infect humans and cause common cold symptoms that are typically cleared but can progress to bronchiolitis or pneumonia^{3,4}. In contrast, MERS-CoV, SARS-CoV-1, and SARS-CoV-2 are more likely to cause severe respiratory disease⁵⁻⁷. While the SARS-CoV-1 (2002/2003) and the MERS-CoV (2012) outbreaks had respective mortality rates of ~10% and 36%^{8,9}, the outbreaks were contained within specific geographic regions with only 8,098 and 2,494 verified infections^{10,11}. SARS-CoV-2 has a lower fatality rate but is more wide-spread than SARS-CoV-1 or MERS-CoV⁹, with 75,704,857 confirmed cases worldwide as of December 21, 2020¹². There are currently over 50 COVID-19 vaccines in clinical trials¹³, and the US Food and Drug Administration (FDA) has authorized the Pfizer-BioNTech and Moderna COVID-19 mRNA vaccines for emergency use^{14,15}. This marks the fastest vaccine development in history, and widespread distribution of an effective vaccine would allow a safer end to stringent, long-term physical

host machinery co-opted for viral replication and signaling pathways that characterize the host response. Proteomic tools can also be used to probe interactions between compounds and proteins, and represent a powerful strategy for drug discovery. This perspective will discuss how proteomics can be leveraged to answer the following open and fundamental questions about SARS-CoV-2 biology. How do the dynamic structures of SARS-CoV-2 proteins and virions inform pathogenicity? What cellular machinery does SARS-CoV-2 utilize during infection? Which signaling pathways are rewired during SARS-CoV-2 infection? What are strategies to monitor COVID-19 pathology and investigate treatment strategies?

How do the dynamic structures of SARS-CoV-2 proteins and virions inform pathogenicity?

Viral proteins dictate the virion's structure and shape, and carry out activities essential for viral replication. Studying the dynamic structures of SARS-CoV-2 proteins and intact virions is essential not only to understand their molecular functions, but also to facilitate the design of effective small molecule therapies that can disrupt virions, viral entry, and virus replication and egress. In addition to atomic-resolution structural approaches that are effective for smaller proteins and protein complexes (e.g. x-ray crystallography and NMR), advances in approaches like cryo-electron microscopy (cryo-EM) have made the analysis of larger, flexible, multi-state proteins and protein complexes more feasible. However, these approaches still require reconstituted complexes often expressed and purified from non-native bacterial or other expression systems, and can require non-native buffers and conditions to achieve structure determination. In contrast, diverse and complementary proteomic approaches including cross-linking mass spectrometry (XL-MS), Hydrogen/Deuterium exchange mass spectrometry (H/DX-MS), and intact protein mass spectrometry can illuminate structural features of proteins and protein complexes under near physiological conditions or even inside biologically-relevant living cells or intact virions (Figure 2)²⁷. Unlike NMR or X-ray crystallography, these mass spectrometry (MS)-based proteomic structure techniques require relatively lower amounts of protein samples. Despite their benefits, XL-MS, H/DX-MS and nMS have been widely considered to be very niche with their applications requiring a high degree of specialized expertise and equipment not generally applied in standard MS experiments. Though there have been great advances in each field opening the technology to non-specialists, this has limited their wide-spread adoption, particularly in rapid-response research. Still, each methodology provides a unique perspective, and when integrated with other structural techniques can provide a more comprehensive understanding of the dynamic complex structures essential for SARS-CoV-2 replication.

Cross-linking mass spectrometry (XL-MS)—XL-MS is a powerful approach that can probe protein-protein interaction (PPI) networks and interfaces while overcoming a number of structural biology limitations^{28,29}. Primarily this includes the ability to: 1) capture structural information from transient and dynamic PPIs and PPI binding interfaces; 2) accommodate not only sample impurities but also structural heterogeneity, flexibility and subunit composition; and 3) be applied to live cell and *in vivo* applications²⁹. When combined with integrative modeling approaches (see below), XL-MS can provide distance restraints for dynamic structure determination. In recent years, a number of

diverse XL-MS strategies have emerged though they all generally rely on chemical cross-linkers to covalently bridge adjacent proteins via reactive amino acid residues, followed by MS-based identification of cross-linked peptides^{29–31}. Cross-linked peptide searching and identification is complicated by two main challenges: 1) the lower frequency of cross-linked peptides in a complex mixture of mainly unmodified peptides; and 2) the complexity of cross-linked peptide spectra which has two covalently linked contributing peptide sequences^{32–34}. The majority of XL-MS strategies include some form of enrichment step to prioritize identification of cross-linked peptides either by their size, hydrophobicity, ion mobility, or charge. Improved software and the development of functionalized chemical cross-linkers that are isotope-coded, MS-cleavable, or contain a reporter ion, have made the identification of cross-linked peptides more accurate and straightforward. While the scope of this perspective is not a comprehensive review of XL-MS approaches, recent in depth reviews of these methods can be found in^{29–31}.

The practical applications for XL-MS vary from informing large macromolecular complexes^{35,36}, to conformationally flexible complexes^{37,38}, to more recent applications probing dynamic interfaces of challenging pathogen-host complexes³⁹ and even virus-like particles (VLPs)⁴⁰. While cryo-tomography and cryo-EM have become very powerful techniques for visualizing viral protein interactions and intact viral particle structures, atomic resolution of dynamic interactions is still challenging. Cryo-electron tomography (cryo-ET) shows ordered binding of part of the flexible receptor to the viral surface, with distal domains in multiple conformations. Using cryo-ET, Meyer *et al.* demonstrated the binding of adeno-associated virus 2 (AAV2) VLPs to the cell surface receptor AAVR⁴⁰ and by complementing with XL-MS data they were able to identify regions of AAVR in close proximity to AAV2 proteins. XL-MS data validated the localization of the PKD2 domain of AAVR, improved the placement of the PKD1 domain of AAVR, and explained the disordered EM density in the structure. By combining cryo-ET, cryo-EM, and XL-MS, collective limitations in size, scale, and resolution could be overcome to determine the structure of AAV2 bound to AAVR soluble domains and thus make biological predictions for the effects of neutralizing antibodies. Prchal *et al.*, combined affinity purification, XL-MS, and NMR data to determine the structure and map interaction interfaces between the cytoplasmic tail of Mason-Pfizer monkey virus (M-PMV) envelope and matrix proteins⁴¹. Similarly, XL-MS was recently leveraged to study how the HIV protein Nef targets surface receptor CD4 for endocytosis to promote HIV infection³⁹. XL-MS data captured flexible and unresolved components of the Nef-CD4-AP2 crystal structure, and confirmed the observation that Nef serves as a flexible connector between CD4 and clathrin AP2 to promote endocytosis and downregulation of CD4³⁹. Finally, XL-MS allows unbiased structure characterization and identification of unknown structural features including additional protein components or post-translational modifications. In Yu *et al.* the thiol-cleavable cross-linker 3,3'-dithiobis(sulfo-succinimidylpropionate) (DTSSP) was used to identify vimentin as a transient interactor of the SARS-CoV-1 Spike (S)-ACE2 virus-host protein complex. Vimentin expressed on the cell surface was found to be important for SARS-CoV-1 virus entry and Vero E6 cells pretreated with antivimentin antibodies showed > 40% reduction in SARS-CoV-1 VLP uptake⁴².

Though to date there have been no published studies using XL-MS to characterize SARS-CoV-2 viruses or viral proteins, the studies above demonstrate some of the potential applications for XL-MS. With the advent of newer and faster instruments, improved XL-MS identification software, and optimized design of new cross-linkers for intra-cellular applications, the reality of large-scale unbiased cross-link peptide identification of whole cell networks is approaching. This type of network and structure data collection is feasible only with XL-MS experiments. One particularly appealing application would be the identification of cross-linked peptides from SARS-CoV-2 infected cells. Comparison of XL-MS data of mock versus SARS-CoV-2 infected cells could illuminate not only the viral-host protein interactions, but also the viral-viral protein complexes and the changes in host-host interactions that occur during infection. By unifying this data with the more standard AP-MS approach, scientists could build not only a network model of SARS-CoV-2 infected cells, but also provide PPI interface data to inform protein complex structures. In addition to exploring SARS-CoV-2 infected cells, researchers could look at the virus itself. The same global XL-MS application to purified SARS-CoV-2 virions could, in one experiment, characterize the virion proteome, identifying both viral and host proteins that make up the virion, as well as provide PPI interface data on virus-virus and virus-host interactions of the virion structure. Combined with sophisticated cryo-EM and cryo-ET images, including recent discoveries made by Yao *et al.*⁴³ and Liu *et al.*⁴⁴, these studies could enhance our understanding of and development of chemotherapies against SARS-CoV-2.

Hydrogen/deuterium exchange mass spectrometry (H/DX-MS)—H/DX-MS measures changes in mass associated with the exchange between protein backbone amide hydrogen and deuterium from the surrounding D₂O. The rate of exchange is dependent on the conformation, surface accessibility, inductive effect of the neighboring groups, stability of hydrogen bonding networks, and the intrinsic chemical properties of the underlying amino acid sequence^{45–48}. It is used to examine conformations of individual proteins or large protein complexes⁴⁹, locate protein sites directly or indirectly involved in binding⁵⁰, probe for allosteric effects⁵¹, monitor the folding dynamics of protein domains⁵², examine intrinsic disorder⁵³ and provide insights into protein-membrane interactions⁵⁴. H/DX-MS is unique in probing conformational states with single-residue resolution. To perform residue-level measurements by H/DX-MS, suitable gas-phase fragmentation of the deuterated peptides by electron transfer dissociation (ETD) or electron-capture dissociation (ECD) is generally applied^{55,56}. Both ETD and ECD generate fragment ions with vibrationally cold energy, minimizing hydrogen scrambling, a phenomenon seen using other fragmentation strategies like collision-induced dissociation (CID)^{57–59}. In 2013, Resetca *et al.* developed a method called Time-Resolved Electrospray Ionization Mass Spectrometry (TRESI-MS) which uses a microfluidic chip in-line with all the steps involved in a ‘bottom-up’ HDX workflow⁶⁰. This development provided faster sample preparation times and improved reproducibility to the point that is now feasible to characterize rapid structural transitions that occur during protein folding⁶¹, ligand binding⁶² or post-translational modification⁶³, applications mostly inaccessible to conventional techniques.

More recently, H/DX-MS has emerged as a potential tool in structural virology exploring the Hepatitis C E2 glycoprotein⁶⁴, influenza hemagglutinin⁶⁵, HIV envelope glycoprotein^{66–69},

and Ebola GP1/GP2⁷⁰. Using H/DX-MS, Ye *et al.*, were able to explore the architecture and self-assembly of SARS CoV-2 N protein and showed that the addition of the C-terminal spacer B/N3 domain to the N2b domain mediates the formation of a homotetramer⁷¹. These types of studies provide insights into the conformational dynamics of proteins in-solution that directly reflect the structural changes, mechanism of viral glycoprotein recognition, and virus neutralization caused by the binding of antibodies and small molecules. Further applications of H/DX-MS for SARS-CoV-2 could help to characterize (1) the effect of post-translational modifications on SARS-CoV-2 protein structures such as glycosylation of S protein or phosphorylation of Orf9b and (2) the binding of small molecules or monoclonal antibodies targeting SARS-CoV-2 proteins, such as S protein. This could not only inform therapeutics, but also provide essential information on host immunity, and potentially the development of different types of neutralizing antibodies.

Intact protein mass spectrometry—While XL-MS and H/DX-MS rely on digesting protein complexes into peptides, intact protein MS enables the analysis of intact proteins and protein complexes. Native MS (nMS) is a unique intact protein-MS methodology that maintains non-covalent interactions and stoichiometry of protein complexes in gas phase^{72,73} while denaturing top-down MS (TDMS) approaches identify proteoforms and PTMs. nMS combined with collision-induced dissociation (CID) disrupts non-covalent interactions between protein subunits based on their strength, and can help decipher stoichiometry and topology of protein complexes. Furthermore, applications with ion mobility-MS (IMMS) have allowed the field to explore greater structural details of large macromolecular assemblies (for detailed review see⁷⁴). Importantly, IMMS allows researchers to separate and characterize protein complexes and protein subunits in gas-phase based on their size and shape. It opens up avenues for structural analysis of heterogeneous protein complexes allowing assessment of stoichiometry, topology, and cross-section of the assemblies and their subunits. IMMS can aid in generating hypotheses about complex structures, conformations, assembly, and disassembly and offers complementarity in structural biology⁷⁴.

nMS can capture transition events in protein complex assembly and disassembly, and more recently in proteolytic cleavage events like those that are essential to coronavirus replication and infection⁷⁴. As described above and shown in Figure 1, the translated coronavirus polyproteins are processed to produce Nsp proteins. In order to investigate the processing of the SARS-CoV-1 polyprotein nsp7–10 region by Mpro (the main protease), as well as the formation of intermediate products and complexes, Krichel *et al.* used nMS to demonstrate how the *in vitro* cleavage efficiencies resemble limited proteolysis of a folded protein and are influenced by tertiary polyprotein structure⁷⁵. In addition, they identify the hetero-tetrameric Nsp7 and Nsp8 complex as the predominant product, thus providing not only the order of proteolytic cleavage, but the formation of post-cleavage structures⁷⁵. While SARS-CoV-2 S protein and ACE2 are challenging to study by nMS given their extensive glycosylation, nMS with limited charge reduction⁷⁶ provided meaningful information about the complex between ACE2 and the receptor-binding domain (RBD) of the S protein as well as the role of heparin in destabilizing the ACE2/RBD association⁷⁷. Yang *et al.* showed that both short (pentasaccharide) and long (eicosasaccharide) heparin oligomers

form a stoichiometric complex with the RBD, indicating a single binding site which alters the protein conformation and subsequently results in a decrease in its ability to associate with ACE2⁷⁷. This study suggests that nMS might be a powerful method for studying the interaction between drugs and their therapeutic target. In addition to these studies, nMS has been applied to investigate viral particle assembly. While the multistep assembly of multiple identical proteins into an icosahedral virus capsid is poorly understood, using charge detection MS, Pierson *et al.* were able to detect trapped intermediates during the assembly of the hepatitis B virus capsid⁷⁸. Subsequent cryo-EM analysis indicated incomplete capsids rather than aberrant structures suggesting that the observed structures are on-path intermediates⁷⁸. Application of these types of experiments to SARS-CoV-2, particularly if complemented with additional structural MS techniques like *in vivo* XL-MS, could characterize the assembly and structure of SARS-CoV-2 virions.

Monoclonal antibodies (mAbs) that target viral proteins are a promising class of therapeutics against infectious diseases like COVID-19⁷⁹, Ebola⁸⁰, Hendra and Nipah virus infection⁸¹. High-quality characterization of intact mAbs is essential and is commonly performed by top-down mass spectrometry (TDMS). Similar to nMS, TDMS strategies avoid the use of proteolytic enzymes, providing intact masses of the molecules, which can determine the presence of multiple proteoforms^{82,83}. MS/MS fragmentation of the intact proteins and large protein subunits can provide amino acid resolution for interpretation of sequence heterogeneity and presence of PTMs^{83–85}. TDMS-associated technologies have significantly advanced over the past decades^{83,86}, mainly focusing on two areas of development, instrumentation and fragmentation approaches^{84,87}. Newer methods such as ultraviolet photodissociation (UVPD)⁸⁸, ECD⁸⁹, ETD⁹⁰ have radically increased the sequence coverage and PTM information for TDMS experiments. As a result, TDMS has emerged as a powerful means in basic, translational, and clinical research for protein identification and different proteoforms elucidation. Though no studies thus far have capitalized on TDMS for studying SARS-CoV-2 antibodies purified from convalescent sera, the potential exists for this application to aid in the identification of proteins and proteoforms that have higher specificity or neutralization potential which could help in design of monoclonal antibody therapy, or in predicting reaction to new mutations.

Integrating complementary structural data—Complementary data collected from these diverse structural proteomic methodologies can be combined to maximize structural insight through integrative modeling. Integrative modeling synthesizes experimental data from structural, biochemical, proteomic and genetic studies to optimize a comprehensive and accurate model of protein complexes^{91–93}. In this way, integrative modeling represents the most complete understanding of a structure that accounts for all data types. Integrative modeling is particularly powerful when defining macromolecular complexes for which atomic resolution might be feasible for individual or small, inflexible, subunits and sub-complexes, but not for the intact holocomplex. For example, integrative modeling was essential to synthesize cryo-EM, XL-MS, and crystal structure data to determine the dynamic structure of the yeast nuclear pore complex (NPC), a challenging macromolecular membrane bound structure consisting of 500+ proteins⁹¹. While cryo-EM and XL-MS heavily feature in integrative modeling, few studies have capitalized on the combined

strengths of XL-MS, H/DX, and nMS. Particularly useful is the ability to define in-solution structures that are more representative of their native state, and in the case of XL-MS, can be performed in living cells.

To date, there are no integrative structures provided for SARS-CoV-2 viral proteins or complexes. Given the recent emergence of the virus, this can likely be attributed to the specialization and time required by each structure technology as well as for integrative modeling. However, applied structural proteomic technologies could be very powerful in studying SARS-CoV-2, as a number of SARS-CoV-2 proteins and virus-host complexes present structural challenges including size, cellular localization, and flexibility. Full-length SARS-CoV-2 Nsp3 is a large, multi-domain, multi-functional, enzymatic, essential protein, that has yet to be fully structurally characterized. Expression, in cell cross-linking, and purification of Nsp3 from human cells could provide structural information pertaining to adjacent Nsp3 domain residue contacts and could capture Nsp3-host interactors that may be transient in nature. Additional TDMS experiments of Nsp3 purified from human cells could identify essential proteoforms that exist in cells. By combining these data with high resolution X-ray diffraction data of individual domains and H/DX-MS studies probing individual domain interactions, integrative modeling could determine a comprehensive and dynamic structure for Nsp3 as it looks in human cells. Investigating SARS-CoV-2 viral entry with integrative modeling informed by structural proteomics like XL-MS could also be particularly powerful to study S protein interactions and SARS-CoV-2 VLP interactions with the ACE2 receptor and the TMPRSS2 protease, as was demonstrated for AAV2 and AAVR in the example above⁴⁰. This type of XL-MS data would pair well with the nMS study by Yang *et al.* described above that focused on defining the ACE2-RBD S protein interaction. Combined with H/DX-MS studies that help identify solute exposed surfaces of free S protein, and protected interfaces of S bound to ACE2 or TMPRSS2, an integrative model could provide useful information about the structure in solution. Understanding the structure of these viral-host interaction interfaces will be critical for developing drugs that disrupt and prevent SARS-CoV-2 entry into host cells.

What cellular machinery does SARS-CoV-2 utilize during infection?

Like all viruses, SARS-CoV-2 does not encode all of the machinery required for its replication, and must co-opt host machinery for the production of progeny virions. Studying viral-host PPIs can identify essential host factors and provide mechanistic details into the viral life cycle (Figure 2). Affinity purification mass spectrometry (AP-MS) is the most widely applied proteomics method to systematically characterize host-viral PPIs by expressing and purifying individual affinity tagged viral proteins in host cells. As a complementary method to classical AP-MS, proximity-dependent labeling (PDL) coupled to quantitative MS (PDL-MS) has emerged to study proximal PPIs, particularly those that are more transient or weaker interactions⁹⁴. While AP-MS and PDL are powerful approaches to define host interactions of single viral proteins, recent developments in complex centric proteome profiling (CCPP) allow global mapping of cellular macromolecular complexes^{95,96}.

Affinity purification-mass spectrometry (AP-MS)—AP-MS is an established method that has been widely applied to systematically characterize host-viral PPIs by expressing individual affinity tagged viral proteins in host cells, purifying stably-bound host protein interactors, and identifying those interactors by MS^{97–103}. In a recent AP-MS study, Gordon *et al.* expressed and purified 26 of the 29 SARS-CoV-2 proteins from HEK293T cells, which led to the discovery of 332 virus-host PPIs¹⁰⁴. Subsequent chemoinformatic analysis identified 69 drugs and small molecules that target the SARS-CoV-2-human PPI network, thus having the potential to disrupt host factor-dependent viral processes. Of 47 compounds tested, two classes of small molecules emerged that exhibited strong antiviral effects: those that modulate the activity of Sigma receptors, and those that inhibit mRNA translation. In a subsequent study, AP-MS efforts in HEK293T cells were expanded to map the full interactomes of SARS-CoV-1 and MERS-CoV¹⁰⁵. Virus-host interactions for each of the three highly pathogenic human coronavirus strains were compared to identify and understand pan-coronavirus molecular mechanisms which revealed a high number of shared interactions between SARS-CoV-1 and SARS-CoV-2 and higher divergence comparing SARS-CoV-1 and 2 to MERS¹⁰⁵. One notable example is the mitochondrial outer membrane protein Tom70 which interacts with the mitochondria localized Orf9b for both SARS-CoV-1 and SARS-CoV-2¹⁰⁵. MERS does not have an Orf9b protein and thus did not show an interaction with Tom70. Interestingly, in the same study Tom70 was validated to be a host-dependency factor for SARS-CoV-2¹⁰⁵.

In another impressive large-scale study that integrated AP-MS and global proteomic data, Stukalov *et al.* systematically mapped the virus-host interactions for both SARS-CoV-2 and SARS-CoV-1 in A549 lung carcinoma cells¹⁰⁶. Performing global protein abundance, phosphorylation, and ubiquitylation profiling of cells overexpressing individual viral proteins, and integrating this data with SARS-CoV-2 PPI networks identified cellular pathways regulated by viral proteins¹⁰⁶. This integrated PPI network was then used to predict well-characterized selective drugs that could be targeted for host-directed therapies and identified drugs targeting AKT and matrix metalloproteases as having anti-SARS-CoV-2 activity¹⁰⁶. These studies demonstrate the potential of rapidly translating AP-MS data into druggable host factors with subsequent identification of repurposable drugs that have potent antiviral activity. Beyond the translational potential of global host-pathogen interaction maps, these studies also show the impact and breadth of work that can be carried out by rapid response of international collaborations between interdisciplinary scientists.

Proximity-dependent labeling (PDL)—Proximity-dependent labeling (PDL) relies on enzymes which catalyze covalent transfer of biotin or biotin derivatives to proteins in proximity of the enzyme, including promiscuous biotin protein ligases (BirA/BioID/TurboID)^{107,108} and engineered ascorbic acid peroxidase (APEX)¹⁰⁹. To map PPIs using PDL, the enzyme is fused to a protein of interest, such as a viral protein, and upon addition of a substrate the proximal proteins will become biotinylated, followed by their enrichment using Streptavidin resin, and analysis using quantitative MS. The covalent labeling of proximal proteins in cells allows for the capture and identification of not only stable, but also transient and weaker interactions, allows purification under harsh lysis conditions, and provides additional spatial information about the subcellular location of

the PPIs through proximal labeling. This was demonstrated for example by applying the APEX-based PDL approach to study agonist-induced changes of G-protein coupled receptors (GPCRs) interaction networks, which successfully characterized functionally relevant GPCR interactors with temporal and spatial resolution¹¹⁰.

PDL has also been extended to study host-pathogen interactions. Coyaud *et al.* applied a combined AP-MS and BioID approach to systematically map the Zika-host interactome composed of over 1200 Zika-host interactions, which revealed extensive organellar targeting by Zika virus and a role of peroxisomes for Zika virus infection¹¹¹. A recent study introduced BirA into the viral genome of the MHV (murine coronavirus) to define the microenvironment of the RTC¹¹². The study identified more than 500 proteins in proximity to the RTC and discovered a close association of viral RNA synthesis sites with the host translation machinery¹¹². Another PDL-MS study performed BioID for 27 SARS-CoV-2 proteins fused to the miniTurbo enzyme in a lung adenocarcinoma cell line¹¹³, which revealed proximal interactions with 2242 host proteins. The inclusion of several host subcellular markers as baits provided testable hypotheses regarding the functional consequences of virus-host interactions. For example the proximal interactome of the N protein revealed the depletion of proteins critical to the formation of stress granules, suggesting that the interaction between N protein and G3BP1 might prevent stress granule formation¹¹³.

These studies demonstrate the power of PDL-MS as a complementary approach to AP-MS to map SARS-CoV-2-host interactions, particularly to characterize transient interactions and inform cellular location. SARS-CoV-2 relies on organellar targeting with replication taking place in membrane bound vesicles derived from the ER and Golgi. Application of PDL to key viral proteins such as members of the RTC (Nsp7, Nsp8, Nsp12, and Nsp14), or key host factors such as the Nsp6 interactor Sigma-1 receptor in the context of SARS-CoV-2 infection could track PPIs and their cellular location, essentially providing an intra-cellular GPS for virus-host PPIs throughout the viral lifecycle. As a recent extension of proximity labeling approaches, split enzymes have been engineered which enable contact-specific PDL. The two fragments of the split enzyme remain inactive apart, and become active upon reconstitution when they are driven together by PPIs^{114,115}. These sophisticated approaches could be particularly powerful in validating and functionally characterizing high-confidence host-viral PPIs identified by AP-MS and/or PDL-MS. A split enzyme PDL experiment could simultaneously validate a putative interaction, determine additional protein complex-specific subunits, and derive intracellular spatial information for the specific interaction. For example, splitting the labeling enzyme between a viral protein and an ER protein can inform protein interactions that take place on the surface of double-membrane vesicles of SARS-CoV-2 RTCs¹¹⁶.

Complex centric proteome profiling (CCPP)—In complex centric proteome profiling (CCPP), samples are gently lysed to preserve native protein complexes which are then: (1) separated into fractions using size exclusion chromatography; (2) digested into peptides and analyzed by highly sensitive data-independent acquisition (DIA) quantitative MS; and (3) protein patterns across the fractions correlated to determine the composition of protein complexes. This approach has recently been utilized to quantitatively compare

protein complexes in two distinct cell-cycle states, suggesting a model for disassembly of the nuclear pore complex during the transition from interphase to mitosis⁹⁶. Host complexes are similarly disassembled and rearranged during viral infection, and CCPP would allow the capture of those changes during SARS-CoV-2 infection in a systematic fashion. Indeed, one of the limitations with AP-MS and PDL-MS strategies, which rely on individually tagged bait proteins, is that the captured PPIs are missing the context of the full suite of viral proteins in infected cells. Therefore, virus-host interactions dependent on multiple viral proteins, nucleic acids, or signaling will be absent in the final analysis. Characterizing SARS-CoV-2 infected cells using the CCPP would avoid the necessity for tagged components, and can provide a global picture of the complexes viral proteins form and interact with, as well as how they manipulate cellular machinery through recruitment or dissociation of specific components.

The integration of CCPP with organelle fractionation could illuminate additional spatial information of host-pathogen interactions^{117–119}. Organelle fractionation alone was recently applied to determine targeting of viral proteins to distinct organelles and to define alterations in organellar proteome composition during Cytomegalovirus infection¹¹⁷. Proteins localized to one compartment in uninfected cells and a different compartment during infection were identified as translocated proteins, with most translocations occurring between the plasma membrane, ER, golgi, and lysosome. Notably, this approach identified MYO18A as a protein that translocates from the plasma membrane to the lysosome during Cytomegalovirus infection, and siRNA knockdown of MYO18A significantly decreased virion production. Because SARS-CoV-2 replication relies on trafficking throughout cellular compartments, especially the ER and Golgi, CCPP combined with organelle fractionation could represent a particularly powerful approach to identify how host proteins are globally hijacked and translocated throughout the SARS-CoV-2 lifecycle.

Towards an integrated virus-host PPI network—Complementary data from various viral-host PPI mapping approaches can be integrated to provide a model for how SARS-CoV-2 hijacks host machinery to replicate inside of cells. A model that accounts for the molecular players involved as well as the timing and localization of events throughout the viral life cycle will aid in the rational design of novel drugs, the repurposing of existing drugs, and the development of combinatorial therapies (Figure 2). Given the initial successes of host-pathogen interaction mapping in standard, though less physiologically-relevant cell lines like HEK293T cells, it is imperative to expand these efforts to clinically-relevant cell models that mimic the infection in human lung epithelia. While expression of individual affinity-tagged viral proteins allows high-throughput discovery of stable virus-host interactions, this approach will miss highly transient or less stable interactions, as well as those interactions that rely on simultaneous expression of multiple viral proteins, such as the viral RNA-dependent RNA polymerase holoenzyme responsible for viral genome replication. Therefore, host-pathogen interaction studies should be performed in the context of viral infection. In addition, cross-linking coupled with AP-MS, or the application of global XL-MS experiments, can capture transient and weaker interactions from SARS-CoV-2 infected cells (see **XL-MS** section above). These experiments can provide PPI network data and inform virus-host protein complex structures. Furthermore, exciting work

could be done to investigate not only SARS-CoV-2, but other human coronaviruses (i.e. HCoV-OC43, SARS-CoV-1 and MERS-CoV) in reservoir and cell lines from cross-species, such as bats. Through identification of conserved virus-host interactions across different coronavirus species, host factors that increase or mitigate pathogenicity, severity, infectivity, and cross-species barriers could be identified. These data would be a starting point for designing pan-Coronavirus treatment strategies.

Which signaling pathways are rewired during SARS-CoV-2 infection?

During infection a complicated battle takes place wherein viruses must simultaneously rewire cellular pathways they need for replication while evading the host cell's innate immune defenses. Perturbations to post-translational modifications (PTMs) allow viruses to quickly manipulate the host environment to control cell cycle, prioritize transcription and translation of viral products, and evade the immune response. For example, HIV-1 Vif degrades regulatory subunits of the key cellular phosphatase PP2A, which results in hyperphosphorylation of many cellular proteins, including substrates of the aurora kinases, and causes cell cycle arrest in G₂^{120–122}. Nuclear factor κB (NF-κB) is an innate immune pathway regulated by phosphorylation and ubiquitylation and has been co-opted by viruses such as HIV-1, which uses NF-κB as a transcription factor to express viral mRNA¹²³. Phosphorylation is also key for viral trafficking throughout the viral life cycle¹²⁴. For example, Ebola entry requires phosphoinositide-3 kinase signaling¹²⁵, Influenza replication relies on nuclear-cytoplasmic shuttling regulated by multiple phosphorylation sites on its nucleoprotein¹²⁶, and the phosphorylation of HIV protein p6 is required for virion budding and release from the cell¹²⁷. Given the important role of perturbing phosphorylation for viral infection, mapping phosphorylation events to kinases and phosphatases could allow for the identification of druggable targets and repurposing of FDA approved drugs¹²⁸.

Ubiquitin signaling is another important PTM for viral replication, and inhibition of the host ubiquitin-proteasome system (UPS) can lead to restriction of viral entry and expression of viral proteins, as it has been shown for coronaviruses including mouse hepatitis virus and SARS-CoV-1¹²⁹. Viruses often exploit the host UPS is to target restriction factors, or host factors that inhibit viral infection, for degradative ubiquitylation. One such example is HIV-1 Vif, which together with the transcriptional cofactor CBFβ hijacks the Cul5-RING E3 ubiquitin ligase and acts as a non-native substrate adaptor to target cellular APOBEC3 restriction factors for polyubiquitylation and proteasomal degradation^{130–137}. Unbiased proteomic approaches that selectively enrich and identify specific PTMs like phosphorylation and ubiquitylation can study global perturbations during infection and highlight cellular signaling pathways that are critical for SARS-CoV-2 replication (Figure 2).

Global phosphoproteomics—Phosphorylation is a quick, reversible PTM that viruses use to (1) regulate viral proteins and (2) alter the stability, subcellular location, and enzymatic activity of host proteins to aid viral replication. Protein phosphorylation is regulated by kinases and phosphatases, which phosphorylate and dephosphorylate substrates, respectively. While serine, threonine and tyrosine are the most commonly phosphorylated and studied amino acids, other amino acids such as arginine, lysine, aspartic

acid, glutamic acid, histidine and cysteine are also phosphorylated and though harder to study, have been implicated in various host biology^{138,139}.

MS-based proteomics has emerged as the method of choice to systematically study protein phosphorylation and its dynamics. To obtain higher sensitivity in identifying phosphorylation sites in complex protein samples such as cell lysates, several approaches have been developed to enrich phosphorylated peptides based on ion metal affinity chromatography, ion exchange chromatography, and antibody-based immunoprecipitation¹⁴⁰. Combined with developments in MS and computational approaches to identify, quantify and localize phosphorylation sites, tens of thousands of phosphosites can routinely be accurately quantified in a single experiment^{141–143}. The ability to subsequently map the quantified phosphorylation events to kinases might reveal druggable targets and the repurposing of kinase inhibitors that have been approved for the treatment of other diseases such as cancer^{144–146}. These inhibitors can then be readily tested for efficacy against viral infection and contribute to a rapid development of efficient treatment strategies for the SARS-CoV-2 pandemic.

The feasibility of this concept was recently demonstrated by applying global phosphorylation analysis to SARS-CoV-2 infected ACE2-expressing A549, Vero E6, and Caco-2 cells^{106,128,147,148}. The study in Vero E6 cells identified amongst others p38, CK2, CDKs, AXL, and PIKFYVE as dysregulated kinases and demonstrated that their pharmacological inhibition restricted SARS-CoV-2 replication¹²⁸. Bouhaddou *et al.* discovered that CK2 is activated by the N protein resulting in upregulation of CK2 cytoskeleton-related targets, which contributed to the formation of filopodial protrusions where virus particles seem to be budding from¹²⁸. Klann *et al.* discovered not only extensive rearrangements of growth factor receptor (GFR) signaling, but also validated antiviral efficacy for multiple GFR inhibitors, thus demonstrating the central function of GFR signaling in SARS-CoV-2 infection¹⁴⁸. Stukalov *et al.* followed a conceptually similar approach integrating global protein abundance, phosphorylation and ubiquitination data measured throughout SARS-CoV-2 infection in ACE2-expressing A549 lung carcinoma cells to identify regulation of cellular pathways perturbed by the virus¹⁰⁶. Targeting selected pathways with drugs they identified several kinase and Matrix metalloproteinase inhibitors with significant antiviral effects against SARS-CoV-2¹⁰⁶.

Global ubiquitylation profiling—Ubiquitylation is another reversible PTM which is essential for the replication of many different virus families. As viruses do not typically encode their own ubiquitin machinery, members of most virus families exploit their host's UPS to ubiquitylate or de-ubiquitylate proteins to target them for proteasomal degradation, alter their trafficking, or to change their activity^{149,150}. Ubiquitylation requires E1 enzymes for activation, E2 enzymes for conjugation, and E3 ligases that recruit the protein substrate. Substrates can be mono- or poly-ubiquitylated, and poly-ubiquitin chains can be connected by any of ubiquitin's seven lysines (K6, K11, K27, K29, K33, K48, and K63) or N terminus. Different linkages allow for different signaling outcomes, for example, K48 linkage is associated with degradation by the proteasome, while K63 linkage is associated with non-degradative signaling including DNA damage repair, innate immunity, and intracellular trafficking. Cells encode a large number of deubiquitylating enzymes (DUBs) that catalyze

the removal of ubiquitin from target substrates, and are important for regulating a number of cellular processes and have been implicated in a number of human diseases^{151–154}. Viruses may hijack E3 ligases to establish novel ubiquitylation events or hijack DUBs to remove established ubiquitylation events in order to aid their replication.

Since enrichment increases the identification of ubiquitination sites, the most commonly used method for assessing global ubiquitination relies on immunoprecipitation of peptides containing a lysine residue modified by diglycine, an adduct left at sites of ubiquitination after trypsin digestion^{155,156}. To distinguish between ubiquitylation events that target proteins for degradation or change protein activity, global ubiquitylation enrichment can be combined with (1) proteasome inhibition which should stabilize proteins targeted for degradation and (2) global abundance proteomics which should identify proteins that are targeted for degradation as downregulated. In contrast to phosphorylation where many enzyme-substrate relationships are well characterized, ubiquitin ligase-substrate relationships are less defined. This complicates the mapping of ubiquitin ligases to ubiquitylation events, and hampers the identification of ubiquitin-related druggable targets. However, ubiquitylation involves a physical interaction between a virus and one or more host proteins, therefore global ubiquitin profiling can be combined with AP-MS and/or proximity labeling to identify relevant interactions with ligases, representing druggable target candidates.

Global ubiquitylation combined with global protein abundance analyses of ACE2-expressing A549 cells infected with SARS-CoV-2 identified virus-mediated ubiquitylation events and host proteins targeted for degradation¹⁰⁶. In this study, 1053 host and viral proteins, including the ACE2 receptor protein, were identified as having SARS-CoV-2-regulated ubiquitylation site. In addition to global ubiquitylation analysis, complementary approaches have suggested ubiquitin signaling as particularly important for SARS-CoV-2 infection. It has been shown by AP-MS that multiple E3 ubiquitin ligases physically interact with viral proteins, which have been subsequently validated to be functionally relevant for SARS-CoV-2 infection. ORF10 was found to interact with the CUL2^{ZYG11B} E3 ligase complex¹⁰⁴ and knockdown of ZYG11B in Huh-7.5 hepatoma cells resulted in decreased SARS-CoV-2-induced cytopathic effect¹⁵⁷, suggesting that ZYG11B is a dependency factor for SARS-CoV-2 infection. Another example is the E3 ligase MIB1, which was discovered as an interactor of Nsp9¹⁰⁴, and interestingly knockout of MIB1 increased the virus-induced cytopathic effect not only for SARS-CoV-2, but also for HCoV-229E, HCoV-NL63, HCoV-OC43¹⁵⁷. These results suggest that MIB1, which is known to function in TBK1 polyubiquitination, which in turn is a signal integrator of multiple RIG-like receptors and positive regulator of IRF3, might establish an antiviral state that broadly controls coronavirus infection¹⁵⁷. Finally, the viral protein Nsp3 has DUB activity, and its inhibition by small molecules decreases SARS-CoV-2 replication¹⁵⁸.

Global protein abundance profiling and integrating signaling data—Viral perturbations in ubiquitin and phosphorylation signaling have downstream consequences at the protein level, and abundance proteomics is the method of choice to investigate how viral infection globally rewires the host proteome. Host proteins that are upregulated and downregulated during SARS-CoV-2 infection can be key host dependency and restriction

factors that the virus utilizes or evades respectively to promote viral replication. Integrating protein abundance data with changes to protein ubiquitylation and phosphorylation can be an incredibly powerful way to identify not only individual proteins, but cellular pathways targeted by the virus, allowing us to build testable hypotheses of the molecular mechanisms of SARS-CoV-2 infection. Bojkova *et al.* analyzed the proteome of SARS-CoV-2 infected Caco-2 cells compared to mock infection, identified significantly perturbed pathways, and tested the effect of drug inhibition of those pathways on viral replication¹⁵⁹. In one notable example, 25 spliceosome proteins were found to be upregulated during infection, and the spliceosome inhibitor pladienolide B showed antiviral activity¹⁵⁹. To better understand the mechanism of how SARS-CoV-2 rewires signaling pathways, PTM and abundance analysis can be performed after infection with genetic mutant viruses to identify which viral proteins are responsible for rewiring a given pathway. This approach has been applied to HIV to study phosphorylation and ubiquitin signaling associated with accessory proteins Vif, Vpr, and Vpu^{120,122,160}. In applying this approach to SARS-CoV-2, Nsp3 is a promising protein for this type of study due to its DUB activity. PTM and abundance changes identified during infection with wild type SARS-CoV-2 but not with a Nsp3 virus may represent proteins and PTMs regulated by Nsp3. Taken together, phosphorylation and ubiquitylation signaling integrated with protein abundance in SARS-CoV-2 infection is an important area of study to understand how the virus rewires host cells and identify druggable targets.

Despite the promise of data integration, its application for host-pathogen signaling has been difficult in part due to variability in and complexity of the individual datasets and in part due to the challenge of measuring both host and pathogen components (reviewed in ¹⁶¹). While identical time points or even split samples might be used to reduce reproducibility issues across data types, there will still be missing values that contribute to noise and inconsistency. In addition, a single protein might have multiple isoforms and multiple phosphorylation or ubiquitylation sites, with each potentially being unchanged, up- or down-regulated, in opposing ways. How to address this nonlinear integration is just the first step. Interpreting integrated data can also be challenging, as tracking meaningful changes across thousands of proteins, and forming testable hypotheses can lead to over- or under-interpretation. Finally, testing the importance of identified proteins or PTMs can be difficult. Genetic manipulation through knock-out, knock-down, or mutation can be complicated by cellular and molecular redundancies. Additionally, to validate the specific modified site, site directed mutagenesis of endogenous genes needs to be performed, which is not established widely and in high-throughput fashion. Drug treatment can also complicate interpretation since there can be nonspecific interactions or cell-type related biology involved. Thus a multi-pronged approach combining multiple functional validations can be key, though are often much lower in throughput. For instance, in Bouhaddou *et al.* parallel siRNA knockdowns and inhibitor treatments targeting the same p38 pathway were performed to demonstrate the p38 pathway as an essential signaling pathway for SARS-CoV-2¹²⁸.

What are strategies to monitor COVID-19 pathology and investigate treatment strategies?

Managing the SARS-CoV-2 pandemic requires rapid and reliable diagnostics to trace disease spread, biomarkers to monitor disease severity, efficacious drug treatment to improve patient outcomes, and the development of vaccines to prevent the spread of disease. In addition

to genomic-based approaches, proteomics might aid the high volume of diagnostic testing required for contact-tracing or be utilized to identify and monitor biomarkers of disease severity or treatment response (Figure 2). Improving patient outcomes also requires the identification of safe and effective drug treatments. As mentioned above, studying SARS-CoV-2 replication inside host cells can identify druggable targets. Proteomic technologies can also be employed subsequently to investigate binding of drugs to those targets and reveal potential off-target binding. These technologies include limited proteolysis-coupled mass spectrometry (LiP-MS), thermal proteome profiling (TPP), and activity-based protein profiling (ABPP) (Figure 2).

Targeted proteomics for monitoring disease progression and treatment

response—Most biological samples taken from individuals suspected with COVID-19 are nasopharyngeal swabs, bronchoalveolar lavages, gargle samples, and blood samples, all of which represent complex proteomes with large dynamic range of protein concentrations. Thus a highly sensitive method is required to allow for detection of SARS-CoV-2 proteins in complex diagnostic testing samples. Targeted proteomics approaches in which a predefined set of proteins and their corresponding peptides are selectively and recursively isolated and then fragmented over their chromatographic elution time, offers highest sensitivity and quantitative accuracy¹⁶². Several studies developed parallel reaction monitoring (PRM) assays for the detection of SARS-CoV-2 proteins^{163–166}, which were subsequently tested in dilute patient gargle samples¹⁶⁴, nasopharyngeal swab¹⁶⁶ and bronchoalveolar lavage samples¹⁶⁵. While these studies demonstrated that it is theoretically possible to detect viral proteins in patient samples using targeted proteomics, compared to PCR-based assays that can experimentally amplify signal and have high-throughput efficiency, the proteomic approach is limited by low sample throughput and sensitivity. Given these limitations proteomics approaches are to date not suitable for diagnostic purposes of COVID-19.

However, proteomics approaches might be powerful to study the biological factors contributing to COVID-19 severity and patient outcomes and identify potential predictive biomarkers. Patients with more severe COVID-19 infections accompanied with rapid deterioration of lung function are distinguished by significant immune dysregulation. The biological pathways that drive disease severity and lead to immune dysregulation remain poorly understood. The identification of differentially regulated proteins and pathways in plasma samples derived from uninfected and infected patient groups with varying disease severity (i.e. asymptomatic, moderate, and severe) would not only provide more information about the biological nature of the dysregulation, but might deliver biomarkers for predicting disease severity that can be measured non-invasively. The complexity and dynamic concentration range of the plasma proteome has posed challenges for reproducible and sensitive MS-based plasma proteome profiling¹⁶². However, the performance of MS-based proteomics has matured reaching a sensitivity and dynamic range which allows quantifying 500–700 proteins routinely and reproducibly across large patient cohorts which makes it interesting for biomarker studies^{167,168}. These molecular measurements can then be correlated with clinical parameters to identify predictive biomarkers^{167,168}. Using unbiased proteomics to discover biomarkers for disease severity has the advantage that it might lead

to more specific biomarkers for COVID-19 compared to established serological assays for cytokine and inflammatory proteins.

To identify potential biomarkers of COVID-19 severity, Messner *et al.* developed a lowcost and high-throughput platform which can handle 180 plasma samples within a single day¹⁶⁹. Using patient sera and plasma from hospitalized patients they identified 27 putative proteins that are differentially expressed depending on the WHO severity grade of COVID-19¹⁶⁹. These proteins include complement factors, proteins of the coagulation system, inflammatory modulators, and pro-inflammatory signaling molecules, thus capturing the host response to SARS-CoV-2 infection¹⁶⁹. If validated in large independent patient cohorts, targeted proteomics assays which in contrast to immunoassays do not rely on specific antibodies could be developed rapidly for the proteomic signature to support clinical decision making and monitor treatment response¹⁶².

Limited proteolysis-mass spectrometry (LiP-MS)—Limited proteolysis (LiP) coupled with quantitative mass spectrometry (LiP-MS) is a powerful approach for systematically characterizing protein conformational changes resulting from ligand or protein binding. In LiP, a broad specificity protease used at low enzyme to substrate ratio for a short time digests native protein extracts to generate large polypeptide fragments dependent on the protein's structural properties¹⁷⁰. The structure-specific protein fragments are then denatured and subjected to tryptic digestion to generate peptides that are amenable for MS analysis¹⁷⁰. An aliquot of the protein extract prior to LiP is fully digested with trypsin as a control and compared to the LiP sample¹⁷¹. When analyzed by quantitative MS, the tryptic peptides containing the LiP sites, so called conformotypic peptides, should have a lower abundance in the LiP digested sample compared to the trypsin-only control. Comparison of proteolytic signatures from samples that have been subjected to different conditions allows the identification of protein regions that underwent structural changes as a response to the perturbation¹⁷¹.

Piazza *et al.* developed a chemoproteomics approach to systematically determine metabolite-protein interactions by combining LiP with quantitative MS on a cell extract in the presence and absence of metabolites to assess metabolite-induced structural alterations on a proteome-wide scale¹⁷². The LiP-small molecule mapping (LiP-SMap) approach was applied to *Escherichia coli* to map the metabolite-protein interactions of 20 metabolites in an unbiased manner. This allowed not only the identification of 1,678 protein-metabolite interactions with the majority being novel interactions, but also the determination of the structural regions that are affected by metabolite binding¹⁷² which were found to be in close proximity to the binding site. Recently, the LiP-SMap approach was extended to enable systematic investigation of protein-small molecule interactions in complex eukaryotic proteomes¹⁷³. Due to the higher complexity of the eukaryotic proteomes resulting in the initial discovery of multiple drug target candidates, Piazza *et al.* developed the machine-learning based LiP-Quant workflow which performs LiP on lysate treated with a drug dilution series to score and prioritize target candidates¹⁷³. While the initial LiP-SMap identified 37 putative drug targets for cells treated with Rapamycin, the additional LiP-Quant scoring method could confirm FKBP1A as the highest-ranking candidate protein target¹⁷³. As of this publication, there have been no published studies using LiP-MS to study

SARS-CoV-2 or any recently identified drugs that are in or being considered for clinical trials. However, in light of the ongoing large scale drug discovery efforts for COVID-19, the relatively simple experimental design of LiP-MS and its broad applicability make it an ideal technique to identify cellular targets of existing drugs in clinical trials or for prioritizing drugs or antibodies based on their off-target reactivity.

Thermal proteome profiling (TPP)—Thermal protein profiling (TPP) combines cellular thermal shift assay (CETSA) with quantitative MS^{174,175}. The basic principle of TPP relies on denaturation and aggregation of proteins in cells at their intrinsic melting temperature, which results in solubility changes of proteins with increasing temperatures. By quantifying the abundance of the protein remaining in solution after subjecting cells to heat spanning a predetermined temperature gradient, a melting profile for a protein can be established. Protein conformational changes upon binding of small molecules or other ligands change the thermal stability of a protein and alter the melting profile. The combination of TPP with multiplexed quantitative MS-based proteomics can systematically determine melting profiles and melting temperature shifts upon drug treatment for thousands of expressed proteins simultaneously to discover drug targets and off-target binding.

As a proof of concept, TPP was applied to the promiscuous kinase inhibitors staurosporine and GSK3182571 with a known spectrum of targets, which induced shifts in the melting temperatures of many kinase targets¹⁷⁴. Interestingly, in addition to the proteins that are directly bound by the ligand, downstream pathway members like regulatory subunits of kinase complexes displayed thermal stability shifts, likely as a result of altered post-translational modifications, thus demonstrating that TPP could inform the drug's mechanism of action¹⁷⁴. The application of TPP was initially limited to soluble, mainly cytosolic proteins. However, since many ligand-binding receptors and drug targets represent transmembrane proteins, the method was extended to profile membrane-protein targets of small molecules by addition of a mild detergent during cell lysis which allows solubilization of membranes but does not solubilize protein aggregates¹⁷⁶. While initially only applied to cultured cells, most recently the method has been extended to tissue (tissue-TPP) and blood (blood-TPP) specimens¹⁷⁷. Therefore, target and off-target binding could be characterized in primary *in vivo* studies, which is critical to predict adverse reactions of drugs. Following intravenous administration of the HDAC inhibitor panobinostat, TPP identified Hdac1, Hdac2 and Ttc38 as known targets across all analyzed tissues, including spleen, liver, kidney, and lung. However, different off-target profiles were obtained across the different tissues which could be explained by heterogeneous levels of protein expression and drug exposure comparing the different tissues¹⁷⁷. A recent study applied TPP to understand off-target effects of Remdesivir, a repurposed drug for treating COVID-19 patients, in uninfected HepG2 cells and identified the hexameric AAA+ ATPase Trip13 as a target of Remdesivir¹⁷⁸. Further experiments are necessary to investigate the relevance of the Remdesivir-Trip13 interaction for SARS-CoV-2, and in fact there is currently a lack of evidence that Remdesivir is effective against SARS-CoV-2¹⁸.

Activity-based protein profiling (ABPP)—Activity-based protein profiling (ABPP) is a sophisticated chemical proteomic strategy that can be used to systematically interrogate

cellular enzymes and discover *in vivo* inhibitors of enzymatic activity (reviewed by Niphakis *et al.*¹⁷⁹ and Kahler *et al.*¹⁸⁰). In ABPP, activity-based probes target a specific activity or structural feature of enzyme active sites in order to covalently modify the target with a reporter tag. The activity-based probe will have: 1) a reactive group that forms the covalent bond to the target protein; 2) a binding group that directs the probe to active sites and typically mimics natural substrates; and 3) a reporter/detection tag that allows measurement of the probe labeling. Depending on the assay and experimental design, labeled events can be visualized by fluorescence microscopy, flow cytometry, or SDS-PAGE, detected *in vivo* by radio-isotope detection, or can be enriched by affinity purification and analyzed by MS^{181,182}. Since probe labeling is dependent on active site accessibility, it can differentiate between inactive and active states of selective targets that would otherwise react to the probe, thus providing a unique opportunity to profile cells in various environmental, genetic, or conditional backgrounds. A number of enzymatic activities can be targeted, including cysteine proteases, cathepsins, kinases, metalloproteases, serine proteases, and oxidoreductases^{179,180,183}.

There are two main ABPP strategies that can be employed, those that are chemocentric and focused on the small molecule(s), and a target-centric approach which focuses on the enzyme. Chemocentric ABPP strategies can be used to discover cellular enzymes that are targets of specific drugs. This type of strategy is exemplified in an ABPP-based study that determined prodrug dimethyl fumarate (DMF), and not monomethyl fumarate (MMF) that DMF is converted to, is responsible for blocking activation of primary T cells in human and mice¹⁸⁴. Activity-based probes have also been used to screen for drugs against specific targets. In an example of a target-based strategy, ABPP was used to identify targets of cellular ABHD2, a serine hydrolase involved in immune response, virus replication, and fertility¹⁸⁵. In this study, novel inhibitors of ABHD2 that could be used to modulate sperm fertility were discovered and probed for off-target profiles. As of this publication, there have been no published studies using ABPP to study SARS-CoV-2 or any recently identified drugs that are in or being considered for clinical trials. One of the strengths of ABPP approaches is their applicability to detecting these events in cell models, primary cells, or *in vivo* animal models. Taken together, these approaches are particularly powerful for identifying protein/enzymatic cellular targets of drugs, characterizing and screening drugs against specific targets, and informing drug efficacy and safety as they are equipped to assess interaction and activity of compounds as well as identify off-target effects. While ABPP methods are still very specialized in their broad application, they could provide unique insight to help identify cellular targets of existing drugs in clinical trials or for prioritizing drugs based on their off-target reactivity for treatments of SARS-CoV-2.

Prioritizing candidates from large-scale SARS-CoV-2 drug screens—Large drug screening efforts are ongoing to identify drug candidates that could be repurposed as SARS-CoV-2 antivirals¹⁸⁶. To prioritize drugs that showed promising results in *in vitro* systems for *in vivo* testing, it is imperative to assess target and off-target binding of promising small molecules from large-scale screens before moving them to animal models and testing them in humans. In light of the broad applicability of TPP, LiP-MS and ABPP, these represent individually or in combination promising strategies for small molecule prioritization. To

assess the effectiveness of novel or repurposed drugs for SARS-CoV-2 treatment in a non-invasive manner, drug discovery efforts could be accompanied with biomarker discovery and validation studies relying on global protein abundance and targeted proteomics approaches to identify biomarkers for monitoring treatment response in body fluids.

Conclusion

The SARS-CoV-2 pandemic necessitates the urgent study of viral protein structure, viral replication, viral-host interaction, and host response to gain molecular understanding of pathogenicity and to investigate strategies for treatment options. The field of proteomics is wellpositioned to inform open questions as discussed in this perspective, and indeed there is a precedent of important proteomic contributions in the study of other viral infections including HIV and Influenza, among others. The pandemic has also impressed upon the need for cross-platform collaborations to quickly investigate, translate, and apply proteomic research into clinical outcomes. This type of rapid-response requires cross-discipline expertise and cooperation to not only generate, analyze and integrate the data, but to interpret, functionally validate, and translate findings into actionable hypotheses that can influence meaningful clinical studies¹⁶¹.

Proteomic techniques are strikingly dynamic in the size and scale of the questions they can answer, including the study of viral infection from the structure of a single viral protein to tracking global changes in the human proteome during infection. Beyond size and scale, proteomics can also provide insight into timing and cellular location, which are critical to understanding how to combat SARS-CoV-2 infection and manage COVID-19 pathology. The application of existing proteomic tools to questions of SARS-CoV-2 biology is pressingly important, and this unprecedented pandemic also invokes an opportunity for creativity in developing new tools, and applying and integrating tools in new ways. As proteomic scientists, it is imperative that we contribute our unique proteomic perspective to open questions about SARS-CoV-2 biology, so we can provide an essential complement to studies in other fields as the scientific community works together to meet this monumental challenge. In fact, a number of studies have already demonstrated the impact and breadth of work that can be carried out by rapid response of international collaborations between interdisciplinary scientists.

ACKNOWLEDGMENT

We would like to thank Kirsten Obernier, Alicia Richards, Helene Foussard, and Jeffrey R. Johnson for critical reading and editing the manuscript and the whole Krogan lab for feedback on the structure of the perspective and figure design. Figure 1 was adapted from “Coronavirus Replication Cycle”, by BioRender.com (2020). Retrieved from <https://app.biorender.com/biorender-templates>.

Funding Sources

This research was supported by grants from the National Institutes of Health (P50AI150476, U19AI135990, U19AI135972, R01AI143292 and R01AI120694 to N.J.K., R01AI122747 to R.M.K.), Defense Advance Research Projects Agency HR0011-19-2-0020 (to N.J.K. and R.H.), by the Laboratory for Genomics Research (LGR) Excellence in Research Award (ERA) from the Innovative Genomics Institute at UC Berkeley (Grantnumber 133122P), and by funding from F. Hoffmann-La Roche and Vir Biotechnology and gifts from The RonConway Family to N.J.K.

ABBREVIATIONS

ABPP	activity-based protein profiling
AP-MS	affinity purification-mass spectrometry
CCPP	complex centric proteome profiling
CETSA	cellular thermal shift assay
CID	collision-induced dissociation
COVID-19	Coronavirus Disease 2019
cryo-EM	cryo-electron microscopy
cryo-ET	cryo-electron tomography
DIA	data-independent acquisition
DMF	dimethyl fumarate
DTSSP	3,3'-dithiobis(sulfo-succinimidylpropionate)
E	envelope protein
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
FDA	US Food and Drug Administration
GFR	growth factor receptor
GPCRs	G-protein coupled receptors
H/DX-MS	hydrogen/deuterium exchange mass spectrometry
LiP-MS	limited proteolysis-coupled mass spectrometry
LiP-SMap	limited proteolysis-small molecule mapping
LiP	limited proteolysis
M-PMV	Mason-Pfizer monkey virus
M	membrane protein
MMF	monomethyl fumarate
N	nucleocapsid protein
nMS	native mass spectrometry
NPC	nuclear pore complex
Nsps	non-structural proteins

Orfs	open reading frames
PDL-MS	proximity-dependent labeling mass spectrometry
PDL	proximity-dependent labeling
PPIs	protein-protein interactions
PRM	parallel reaction monitoring
PTMs	post-translational modifications
RBD	receptor-binding domain
RTCs	replication and transcription complexes
S	Spike protein
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
TDMS	top-down mass spectrometry
TPP	thermal proteome profiling
UPS	ubiquitin-proteasome system
VLPs	virus-like particles
XL-MS	cross-linking mass spectrometry

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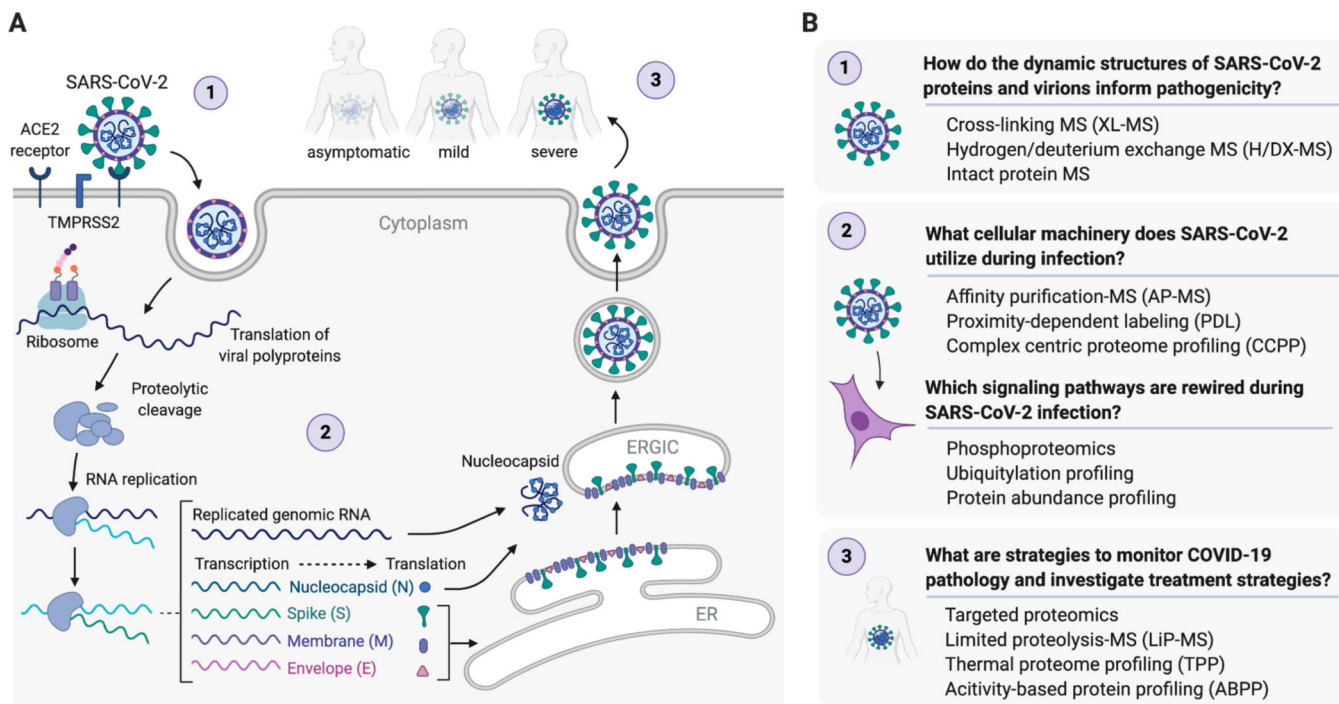


Figure 1. SARS-CoV-2 life cycle (A). Open questions to further our understanding of SARS-CoV-2 biology and proteomic techniques that can be leveraged to address these questions (B).

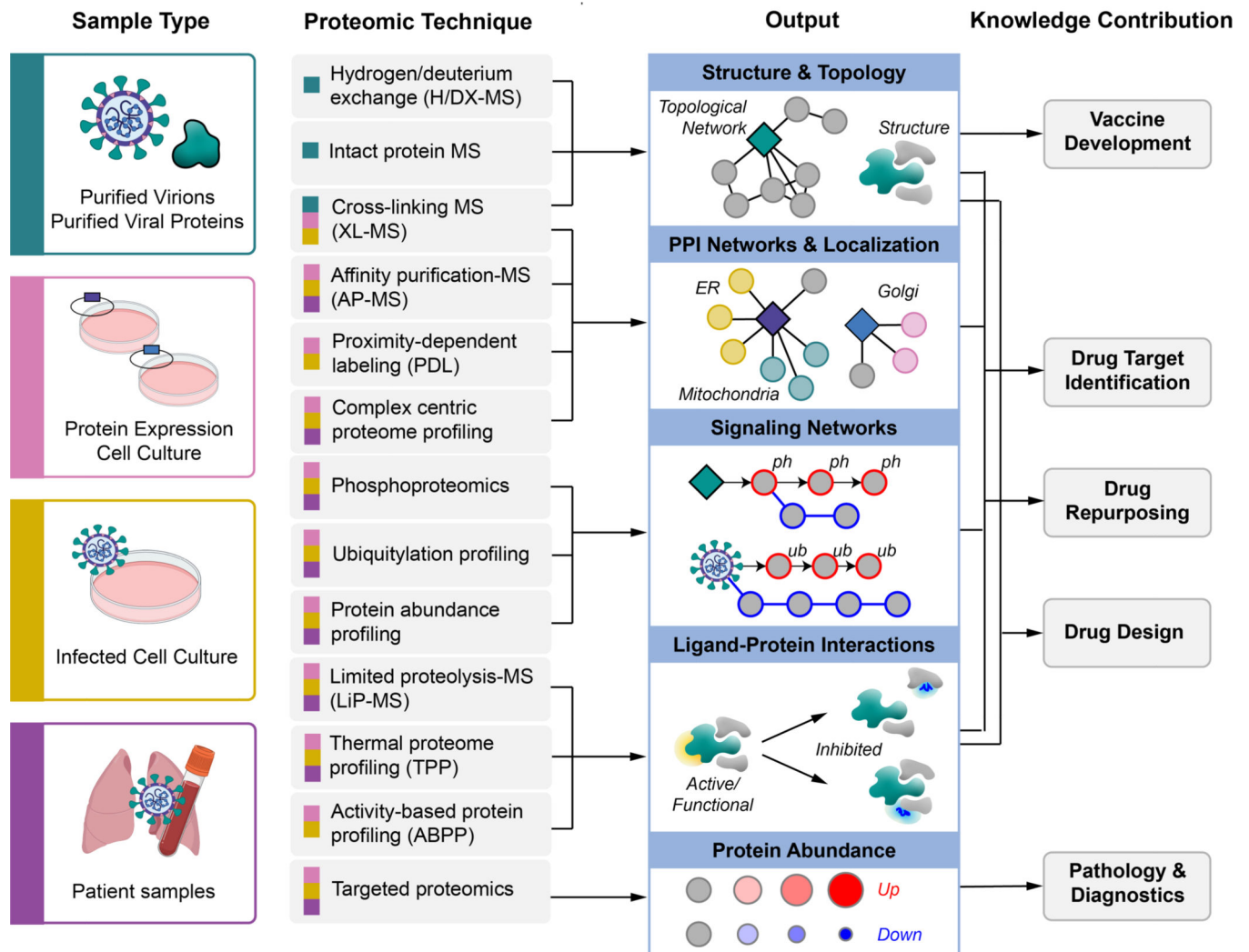


Figure 2. Overview of MS-based proteomics techniques proposed to study SARS-CoV-2, including sample types that can be used as input, molecular insights that can be obtained as output, and how the technologies can be integrated to inform SARS-CoV-2 biology and COVID-19 pathology.

Table 1.

Proteomic studies on SARS-CoV-2 highlighted in the perspective.

Proteomic Technique	Sample Type	Study Objective	Reference
H/DX-MS	Purified protein	N protein structure	Ye <i>et al.</i> Architecture and Self-Assembly of the SARS-CoV-2 Nucleocapsid Protein. <i>bioRxiv</i> 2020. https://doi.org/10.1101/2020.05.17.100685 .
nMS	Purified protein	S-ACE2 virus-host protein complex structure; drug mechanism (heparin)	Yang <i>et al.</i> The Utility of Native MS for Understanding the Mechanism of Action of Repurposed Therapeutics in COVID-19: Heparin as a Disruptor of the SARS-CoV-2 Interaction with Its Host Cell Receptor. <i>Anal. Chem.</i> 2020. https://doi.org/10.1021/acs.analchem.0c02449 .
AP-MS	HEK293T cells expressing SARS-CoV-2 proteins	Virus-host protein-protein interactions; drug candidates	Gordon <i>et al.</i> A SARS-CoV-2 Protein Interaction Map Reveals Targets for Drug Repurposing. <i>Nature</i> 2020. https://doi.org/10.1038/s41586-020-2286-9 .
AP-MS	HEK293T cells expressing SARS-CoV-2, SARS-CoV-1, and MERS-CoV proteins	Virus-host protein-protein interactions; drug candidates	Gordon <i>et al.</i> Comparative Host-Coronavirus Protein Interaction Networks Reveal Pan-Viral Disease Mechanisms. <i>Science</i> 2020, 370 (6521). https://doi.org/10.1126/science.abe9403
AP-MS; phosphoproteomics; ubiquitylation profiling	A549 cells expressing SARS-CoV-2 proteins; ACE2-expressing A549 cells infected with SARS-CoV-2	Virus-host protein-protein interactions; transcriptome, proteome, phosphoproteome, and ubiquitome during infection; drug candidates	Stukalov <i>et al.</i> Multi-Level Proteomics Reveals Host-Perturbation Strategies of SARS-CoV-2 and SARS-CoV. <i>bioRxiv</i> 2020, 2020.06.17.156455. https://doi.org/10.1101/2020.06.17.156455 .
PDL	A549 cells expressing SARS-CoV-2 proteins	Virus-host proximal protein interactions	Samavarchi-Tehrani <i>et al.</i> A SARS-CoV-2 - Host Proximity Interactome. <i>bioRxiv</i> 2020, https://doi.org/10.1101/2020.09.03.282103
Phosphoproteomics	Vero E6 cells infected with SARS-CoV-2	Phosphoproteome during infection; drug candidates	Bouhaddou <i>et al.</i> The Global Phosphorylation Landscape of SARS-CoV-2 Infection. <i>Cell</i> 2020, 182 (3), 685-712.e19.
Abundance proteomics; phosphoproteomics	Vero E6 cells infected with SARS-CoV-2	Transcriptome, proteome, and phosphoproteome during infection	Davidson <i>et al.</i> Characterisation of the Transcriptome and Proteome of SARS-CoV-2 Reveals a Cell Passage Induced in-Frame Deletion of the Furin-like Cleavage Site from the Spike Glycoprotein. <i>Genome Med.</i> 2020, 12 (1), 68.
Phosphoproteomics	Caco-2 cells infected with SARS-CoV-2	Phosphoproteome during infection; drug candidates	Klann <i>et al.</i> Growth Factor Receptor Signaling Inhibition Prevents SARS-CoV-2 Replication. <i>Mol. Cell</i> 2020. https://doi.org/10.1016/j.molcel.2020.08.006 .
Abundance proteomics	Caco-2 cells infected with SARS-CoV-2	Translatome and proteome during infection; drug candidates	Bojkova <i>et al.</i> Proteomics of SARS-CoV-2-Infected Host Cells Reveals Therapy Targets. <i>Nature</i> 2020, 583 (7816), 469-472.
Targeted proteomics	Vero E6 cells infected with SARS-CoV-2	Diagnostic methods	Bezstarosti <i>et al.</i> Targeted Proteomics for the Detection of SARS-CoV-2 Proteins. <i>bioRxiv</i> , 2020, 2020.04.23.057810. https://doi.org/10.1101/2020.04.23.057810 .
Targeted proteomics	Patient samples (gargle)	Diagnostic methods	Ihling <i>et al.</i> Mass Spectrometric Identification of SARS-CoV-2 Proteins from Gargle Solution Samples of COVID-19 Patients. <i>bioRxiv</i> , 2020, 2020.04.18.047878.
Targeted proteomics	Vero E6 cells infected with SARS-CoV-2; patient samples (nasopharyngeal swabs, bronchoalveolar lavage)	Diagnostic methods	Zecha <i>et al.</i> Data, Reagents, Assays and Merits of Proteomics for SARS-CoV-2 Research and Testing. <i>Mol. Cell. Proteomics</i> 2020, 19 (9), 1503-1522.
Abundance proteomics	Patient samples (sera and plasma)	Biomarkers of COVID-19 disease severity	Messner <i>et al.</i> Ultra-High-Throughput Clinical Proteomics Reveals Classifiers of COVID-19 Infection. <i>Cell Syst</i> 2020. https://doi.org/10.1016/j.cels.2020.05.012 .

Proteomic Technique	Sample Type	Study Objective	Reference
TPP	HepG2 cells treated with compounds	Off-target effects of COVID-19 drug candidates (remdesivir, hydroxychloroquine, and more)	Friman <i>et al.</i> CETS MS Profiling for a Comparative Assessment of FDA Approved Antivirals Repurposed for COVID-19 Therapy Identifies Trip13 as a Remdesivir off-Target. <i>bioRxiv</i> 2020. https://doi.org/10.1101/2020.07.19.210492

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