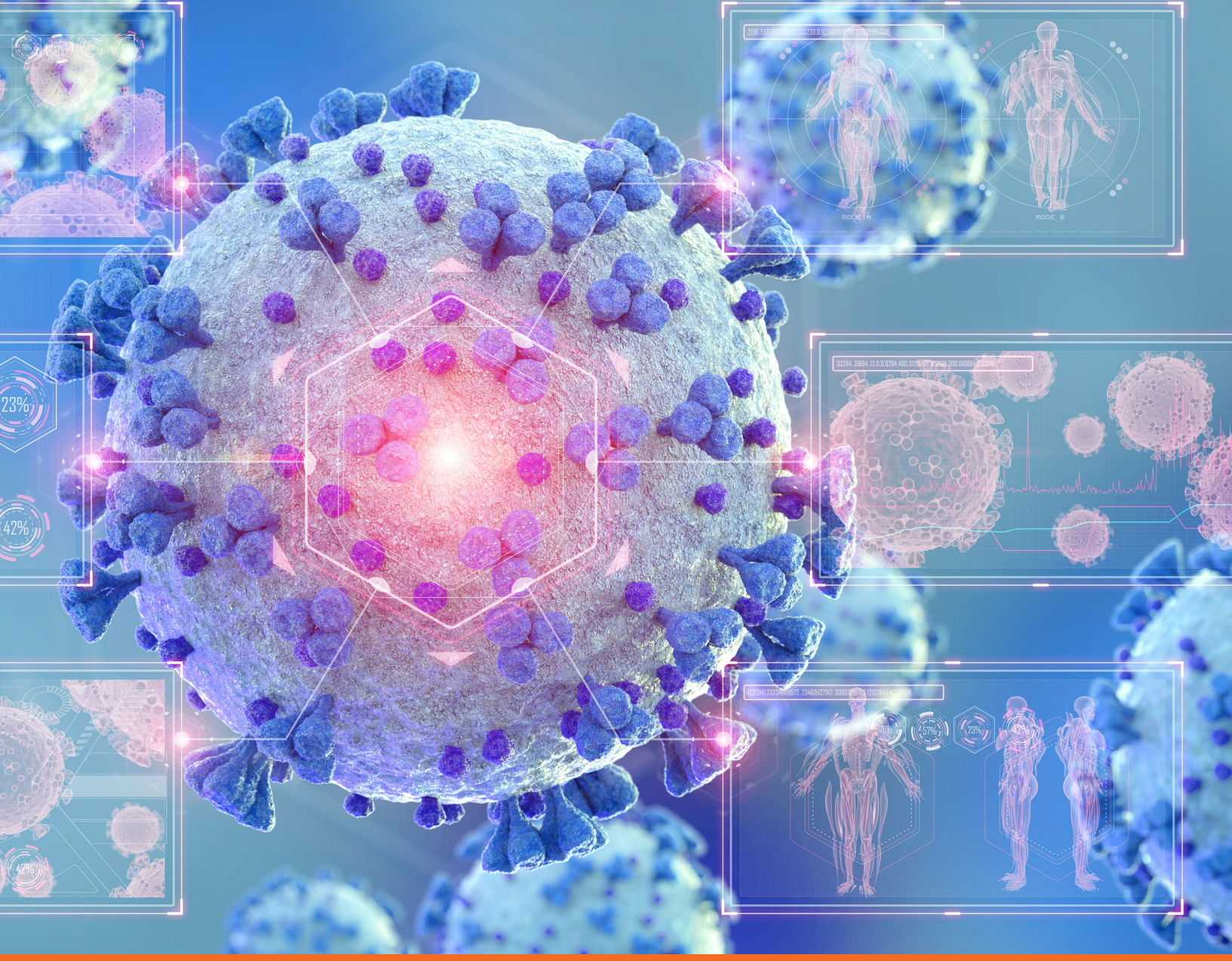

Flow Cell Sorting: Biosafety for COVID-19 Research and Diagnostics

ARTICLE COLLECTION

Cytometry PART A
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Contents

Introduction

The SARS-CoV-2 pandemic has created numerous challenges in the clinical and research communities. Particularly impacted are the operation of shared resource facilities, which provide valuable experimental services and analytical expertise to their users. Conforming to this new working environment has required adaptation and creation of new operating procedures to safely manage the risk of exposure to facility staff from pathogen-carrying samples during handling and processing. Issues pertaining to protecting personnel from exposure to infectious particles, operation and administration of a shared facility under heightened biosafety regulations, the care and use of instruments such as flow cytometers in biological safety cabinets, and safe disposal of waste products all need to be considered.

The goal of this article collection is to present recent guidelines developed from shared resource labs and authorities in the flow cytometry research and clinical setting for navigating operations during the SARS-CoV-2 pandemic. Guidelines for facility operation to sample handling, as well as examination of instrument safety are discussed, and recommendations provided. First, Aspland et al. (2021) provides an overview of considerations for operating a shared resource laboratory during a pandemic. This involves mitigating risks to personnel, alteration in how equipment is utilized in containment, and proper disposal of potentially infectious waste. Second, Charropadhyay et al. (2021) presents a commentary on how flow cytometry can contribute to the study of COVID-19 infection and the immune response to elucidate the mechanisms underlying the disease course. Next, Aspland et al. (2021) evaluates the risk to personnel for exposure to aerosol during operation of flow cytometers at instrument locations identified as 'failure modes' due to instrument specifications or inadequate aerosol containment. Finally, two paired articles provide procedures for safe handling of samples: Cossarizza et al. (2020) presents guidelines for the handling and processing of human blood samples, revised with considerations specific to COVID-19; and Reifel et al. (2020) describes biosafety procedures for processing unfixed sample of infectious materials and provides a series of recommendations to be acted upon.

This article collection serves as a resource for shared resources laboratories charged with processing SARS-CoV-2 samples in either a research or clinical setting. By providing operational guidelines ranging from handling of sample to administrative operation of the facility, we hope this article collection will enhance the safety and efficiency of shared resource laboratories and make them better prepared for future instances of emerging infectious disease.

By Jeremy Petravicz, Ph.D., Editor,
Current Protocols

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SRL COMMUNICATION

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Biosafety during a pandemic: shared resource laboratories rise to the challenge

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Abstract

Biosafety has always been an important aspect of daily work in any research institution, particularly for cytometry Shared Resources Laboratories (SRLs). SRLs are common-use spaces that facilitate the sharing of knowledge, expertise, and ideas. This sharing inescapably involves contact and interaction of all those within this working environment on a daily basis. The current pandemic caused by SARS-CoV-2 has prompted the re-evaluation of many policies governing the operations of SRLs. Here we identify and review the unique challenges SRLs face in maintaining biosafety standards, highlighting the potential risks associated with not only cytometry instrumentation and samples, but also the people working with them. We propose possible solutions to safety issues raised by the COVID-19 pandemic and provide tools for facilities to adapt to evolving guidelines and future challenges.

KEYWORDS
COVID-19, SARS-CoV-2, biosafety guidelines, shared resource laboratory (SRL), pandemic, epidemic, emerging disease, cytometry, flow cytometry

1 | INTRODUCTION

Biohazardous materials are commonly encountered in flow cytometry SRLs. As with any laboratory setting, the standard approach to safety when working with potential hazards is to perform a thorough risk assessment on the infectious agents, reagents, standard operating procedures (SOPs), and the instrumentation proposed for use. Protocols are put in place to help reduce these inherent risks, managed through the implementation of primary controls, such as engineering controls, personal protective equipment (PPE), and SOPs (1). Biosafety considerations when handling samples before, during, and post-acquisition have always been front of mind in flow cytometry SRLs, particularly related to droplet-based cell sorters. The SRL, by its definition, handles a wide variety of samples and hosts users from many laboratories, universities,

All authors contributed equally to this study.

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institutions and companies. Considering that the current pandemic is spread via respiratory transmission and remains viable on surfaces for prolonged periods (2), the actions of one individual can impact many with wide-spread downstream consequences. In times of epidemics, pandemics and emerging disease, the potential risks associated with working within an SRL are evolving, giving cause for re-evaluation of our practices to accommodate these new challenges.

2 | HUMAN-ASSOCIATED RISKS

The SRL, by its nature, is a multi-user environment that facilitates interaction between different members of a research community including SRL staff and users. In the case of COVID-19, a vaccine is currently unavailable against the causative agent (SARS-CoV-2), and it is currently not feasible to perform screening for asymptomatic or pre-symptomatic individuals. This pandemic has necessitated significant changes in the working environment and management of the workforce, with increased expectations put on staff and users. As a result of these changes, added attention needs to be given to the human contribution to the risks associated when working within the context of a shared-use space. With respect to biosafety risks, staff and users must now be included in this assessment and operational guidelines should be identified. For most institutions, these expectations are defined by the level of biosafety threat to individuals and the specific institution's approach to risk (1). We review below some simple strategies that can be employed to maintain a safe and healthy SRL working environment.

2.1 | RISK ASSESSMENT AND CONTACT TRACING

Perhaps one of the biggest changes in the pandemic SRL environment is the potential source of significant biosafety risks. In the pre-COVID-19 era, the focus was on the biosafety risks posed by the samples and reagents brought into the SRL. The standard mitigation approach was a detailed sample-associated biological safety assessment (3) that led to the application of engineering controls, PPE and SOPs for processing and analyzing samples. While communication between investigator, SRL, and safety officer remains critical to ensure a cohesive approach when defining a biological safety assessment in the context of an SRL (Figure 1), the COVID-19 pandemic has added additional considerations to this previously defined process. This global pandemic has caused a paradigm-shift whereby potentially the greatest sources of biosafety risks in an SRL are now the people who enter it; staff, users and external visitors such as field service engineers. The risk posed by an individual carrying SARS-CoV-2 must now be considered and integrated into risk assessments. Any risk assessments should include:

1. Identification of the workforce with potential for exposure—including competency and experience as well as enrollment in medical surveillance.

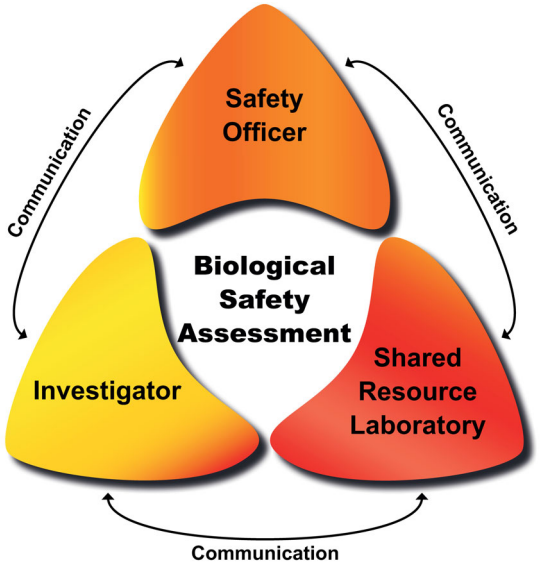

FIGURE 1 Effective communication between investigator, shared resource laboratory, and safety officer ensures a cohesive approach when defining biological safety assessment in the context of an SRL. As in everything we do, our ability to identify the risks, assess them, and then go on to manage them is limited by our ability to communicate with all involved parties. It is in the framing of these biosafety discussions that SRL staff can have the most impact, where the focus is understanding, communicating perceived risks, followed by collaborating to determine an appropriate safety response. While compromise may not always be possible, there are invariably instances where inclusion of users leads to innovative solutions and new approaches to safety. There is a certain amount of trust required between users and SRL staff. This trust is developed by having ongoing discussions around safety, developing a cultural expectation of safety and continued inclusive discussions. There is a significant mental and time burden to the maintenance and communication of appropriate biological risk management. However, it is imperative, especially during pandemics, that SRLs have effective processes in place to ensure the safety of everyone who uses their space [Color figure can be viewed at wileyonlinelibrary.com]

2. Characterization of the risk—including hazards, risk group of the agent, risk of exposure, activities that increase the risk of exposure, and an evaluation/prioritization of the risks.
3. Risk mitigation—including creating mitigation strategies, determining mitigation necessity, communication of strategies to affected personnel, and validation of mitigation strategies.

This new source of risk has necessitated the development of screening mechanisms to identify and exclude potentially infected individuals. These methods can range from high-tech approaches that use purpose-built programs for self-assessment, to low-tech paper versions (4, 5). More detailed screening methods, from sampling of body temperatures, as well as polymerase chain reaction and serological tests, have also been employed. Institutional and regional policies will dictate when this type of testing is warranted and provide guidelines regarding periods for self-isolation or quarantine. In many institutions, once a positive case has been identified, contact tracing is undertaken to identify individuals at risk so that they may follow the

TABLE 1 Software types, applications and important features for facilitating safe work practices during a pandemic

Category	Use cases	Examples Free	Paid	What to look for
Facility management	Bookings, Usage tracking User tracking Record user agreement with entry conditions; update users on changing requirements *Comments: None*	Quartzy (academic and non-profit) (quartzy.com/)	Stratocore (stratocore.com) iLabs (agilent.com/en/products/lab-management-software/core-facility-management) IDEA ELAN (ideaelan.com/) Agendo (agendo.science/) Calpendo (exprodo.com/calpendo)	Control bookings and instruments logins, e.g. require gaps between users Ability to group instruments into sets that cannot used at the same time Approval for bookings Management of safely approvals Document management with user response tracking
Collaborative communications	Shared inboxes allow centralization of email communications with users Mailing list software facilitates mass communications Wiki and blog software provides repository of facility information and communications *Comments: Ticketing systems (helpdesk/servicedesk) systems can also be useful*	Google Groups (groups.google.com)	*Shared Inboxes*: Front (frontapp.com) Gmelius (gmelius.com) *Mailing List Software*: Mailchimp (mailchimp.com) *Wiki/Blog Software*: Confluence (atlassian.com/software/confluence) Wordpress (wordpress.com)	Shared inboxes Shared drafts Assign emails to individuals Open/read tracking
Instant communications	Communication between facility staff Communication between users and staff "crowd-sourcing" support, for example, facilitates expert users helping other users when facility staff are not on-site) *Comments: Many of the commercial products have free tiers that have been expanded during COVID-19*	Slack Google Chat	Slack (slack.com) Microsoft Teams (microsoft.com/teams)	Ability to support multiple organizations, for example, users may already be using a product with other groups and need to be able to quickly switch between accounts
Remote control	Observe and control instrument PCs remotely, for example, for trouble shooting Remote setting of sort regions *Comments: Security is critical when enabling remote access across the internet; look for security focused reviews and seek approval from cyber-security team*	Chrome Remote Desktop (remotedesktop.google.com) No Machine (nomachine.com) MeshCentral (meshcommander.com/meshcentral2)	TeamViewer (teamviewer.com) SplashTop (splashtop.com) Remote Utilities (remoteutilities.com) ConnectWise Control (connectwise.com/software/control)	Multi-factor authentication Support for a wide-range of operating systems
Remote meetings and assistance	Remote meetings and assistance *Comments: Many of the commercial products have free tiers that have been expanded during COVID-19*	Jitsi (meet.jit.si)	Google Meet (meet.google.com) Zoom (zoom.us) GoToMeeting (gotomeeting.com) WebEx (webex.com.) Microsoft Teams (teams.microsoft.com)	Direct use in a browser (no download required) Persistent meeting URLs
Digital check-in and visitor management	Track people who have entered the facility in order to facilitate contact tracing Pre-entry screening questions and reminders *Comments: These products can raise serious privacy concerns that need to be considered in the light of local guidelines or national regulations: for example, see this guidance for Australians—www.oaic.gov.au/engage-with-us/consultations/guidance-for-digital-check-in-providers-collecting-personal-information-for-contact-tracing/*	Google Forms with a QR Code	Swipedon (swipedon.com) Sine (sine.co) COVID19 Tracker (covid247.org)	Ability to pre-screen visitors with questions Mobile apps to facilitate contactless check-in Geofencing for automated cheek-in/out High-resolution tracking (using beacons/tags) to facilitate contract tracking

Additional Notes
Many vendors offer discounted rates/free plans for educational or non-profit use.
Care must be taken when evaluating license agreements, for example, some products may claim to be free for non-commercial use but these free plans do not cover use within an SRL.
There are many review sites that aggregate user reviews for Software-as-a-Service (SaaS) products; for example, getapp.com or capterra.com.

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recommended procedures for testing and self-isolation (6). SRL facility management systems can help to quickly determine who should be contacted when a user or staff member tests positive (7). Some SRLs have access to high-tech methodologies that enable contact tracing such as badge scanners at the door of the SRL. Low-tech solutions should also be considered including having a sign-in/sign-out log. This manual system is important for users who come to the SRL for purposes other than to utilize an instrument, such that usage would not be recorded in instrument booking systems. For further details on possible booking systems and visitor tracking options please refer to Table 1.

Additional consideration should be given to those who must enter the SRL to provide specialist services, such as instrument maintenance and installation. Prior to their arrival, these individuals should be informed of the institution's screening process, escort rules, and other relevant guidelines for working within the facility. In the case where they are arriving from another country or region, government travel regulations must be considered and adhered to.

While every effort can be made to identify all potential contacts of a positive case, this may not always be all-encompassing. Thus, having in place a policy that assumes anyone may be infectious (similar to standard precautions when handling biological samples) is crucial to ensure a safe SRL working environment.

2.2 | Minimizing Transmission

Many facilities have put in place operational policies that help to control the spread of SARS-CoV-2. Although the specific policies and recommendations may vary between institutions, they all serve to reduce transmission through: (1) physical distancing of individuals; (2) improved decontamination of common workspaces; and (3) the use of PPE.

1. *Physical distancing*: There are different methods that can be employed to physically distance users and operators within an SRL. If space is not an issue, instruments can be relocated to other spaces or moved further apart to facilitate physical distancing. Rotating shifts for both core facility staff and users can help to reduce the number of persons in a given lab at one time. To complement this, strategies should be employed to minimize possible overlap of users and the number of users in a space by preventing the simultaneous booking of instruments in close proximity. The companion manuscript on regulatory measures (7) covers these issues in detail. Remote support can further reduce physical contact, while maintaining training and support. These same software solutions can also be employed by users who often will work side by side on an instrument to demonstrate data acquisition to new colleagues. These strategies can be encouraged to allow for mentoring to continue, while taking place remotely. There are a number of easy-to-use software platforms (see Table 1) that can be used to facilitate remote sessions between SRL users and staff alike, as reviewed in detail by Daniels et al. (8).
2. *Environmental decontamination procedures*: Cleaning procedures will vary between facilities; however, these typically include cleaning protocols for high-touch surfaces such as instrument keyboards, mouse, webcams, headsets, as well as all surface areas of the instrument contacted by a user (9–14). A list of surface disinfectants shown to be effective against SARS-CoV-2 can be found in Table 2. Enhancement of ventilation in SRL spaces is also recommended to further reduce the risk of environmental contamination (19, 20). Reducing back-to-back bookings by providing a 15 to 30 min gap between bookings on an instrument allows time for air exchange, sanitization of work surfaces, limits overlap between users and reduces the number of individuals within the SRL at any one time. All of these measures in combination are designed to reduce the concentration of potentially contaminated droplets and aerosols.
3. *Personal protective equipment*: The recommendations for the type of PPE and when to use them vary widely and can be conflicting between institutions and countries around the world. SRLs should refer to and follow the policies as dictated by their own local institutions. Examples of common PPE used in SRL include masks and other suitable face coverings, face shields, disposable gloves, clean lab coats, and safety goggles. Studies have shown effective reduction in the transmission of particulates through the use of masks (21). Various kinds of face shields and masks are available and reduce droplet spread to different degrees as assessed by physical testing (22).

2.3 | Communication

Ensuring consistent uptake of new policies associated with pandemic working conditions, while maintaining strong working relationships requires consistent messaging, support and a good safety culture (1). Institutional policies tailored to the SRL should have the backing of the administration. These policies are best put into place if the SRL defines them in accordance with state and national, as well as institutional guidelines, and acquires approval from institutional administration (Supporting Information Table S1). Having a clear, well-thought-out plan is essential and takes time and feedback from key stakeholders, including SRL staff, biosafety officers, workplace health and safety committee, SRL support committee, and users of the SRL. This inclusion facilitates acceptance of the resulting plan and successful uptake by staff and users. These interactions should be structured with a focus on enabling user compliance (1) and are best supported with imagery, videos, demonstrations, and documentation, all of which help to facilitate the transfer of skills, techniques and ultimately behavioral changes. Structuring a plan that details what is expected, along with the reasons for these changes, and potential consequences, will aid in transitioning to new working conditions. Moreover, reminders of policies can aid in ensuring compliance as working conditions change. This important task could be complicated due to the reduced number of SRL staff at a given time to check that

TABLE 2 Inactivation of SARS-CoV-2 virus by commonly utilized active ingredients

Active ingredient	Surface/sample type tested	Concentration	Time (minutes)	Temperature (°C)	Log reduction	Reference
Ethanol	Hand sanitizer	49% w/w	1	21	≥4.2	(15)
	Surface disinfectant (non-porous)	62%, 70%, 75%, 80%	0.25, 0.5, 1	Room temperature	>4.0	(16)
		95%	0.25, 0.5, 1	Room temperature	>1.0–<3.0	
Formaldehyde	Tissue culture fluid	4%	15, 60	18–25	≥4.8, ≥5.0	(17)
		2%	15, 60	18–25	≥4.8, ≥5.0	
	Infected monolayer	4%	15	18–25	≥6.9 (live virus still detectable)	(17)
		4%	60	18–25	≥7.5	
		2%	15, 60	18–25	≥6.8, ≥7.3 (live virus still detectable)	
Formaldehyde + glutaraldehyde	Tissue culture fluid, infected monolayer	2%+ 1.5%	15, 60	18–25	≥5.0, ≥6.7	(17)
Glutaraldehyde	Surface disinfectant (non-porous)	2.4%	0.25, 0.5, 1	Room temperature	>4.0	(16)
Isopropanol	Surface disinfectant (non-porous)	70%, 75%, 80%	0.25, 0.5, 1	Room temperature	>3.0–>4.0	(16)
Methanol	Infected monolayer	100%	15	18–25	≥6.7	(17)
			30	Room temperature	>4.0	(18)
Para-chloro-meta-xylenol	Hand sanitizer	0.094% w/v	5	21	≥4.7	(15)
Quaternary ammonium compound	Surface disinfectant (non-porous)	0.077% w/w	5	21	≥4.1	(15)
Sodium hypochlorite	Surface disinfectant (non-porous)	0.0525%	0.25, 0.5, 1	Room temperature	>1.0–<3.0	(16)
		0.525%	0.25, 0.5, 1	Room temperature	>4.0	
		0.1%	0.25, 0.5, 1	Room temperature	>4.0	

the SRL room occupancy is correct and everyone works following the "new normality" policies. Encouraging a collaborative culture where users remind each other of the new behaviors can greatly aid in adoption.

The need for physical distancing means the majority of SRLs are operating with some level of remote support. There are added pressures at this time as research groups must keep working, often on rotating shifts, and under the expectation they will not exceed booking times in order to maintain compliance with room occupancy restrictions. This extra pressure may potentially impact users' abilities to correctly follow protocols. Thus, added precautions should be implemented to not only limit the frequency of potential errors, but also to limit their impact. For example, additional training time in the form of remote support by SRL staff can be included in the first few sessions a new user runs on their own. This also means that users should be trained to follow all new procedures and consistent communication should be implemented to support users in these new policies (1).

The strategies reviewed here aim to reduce person-to-person contact and subsequent spread of disease while maintaining interactions between members of an SRL. For SRLs, the challenge is to implement working policies that both safeguard the health and well-being of all staff and users, while maintaining a high level of support to ensure continuity of research services. This is a delicate balance as the measures put into place to reduce person-to-person contact can potentially also reduce the ability for SRL staff to provide support to their users. An example of a risk assessment for working with a SARS-CoV-2 infected user in an SRL setting is provided in Appendix.

3 | INSTRUMENTATION AND INHERENT RISKS

Historically, facilities have effectively managed inherent risks by implementing "Standard Precautions" in laboratories in line with their biosafety containment level. Standard Precautions are such that all

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human specimens are assumed potentially infectious, and protective measures are implemented to reduce the risk of transmission (23). These precautions include the use of protective barriers such as: hand hygiene, gloves, gowns, masks, and protective eyewear or face shields. If procedures are likely to result in a higher risk of transmission, for example, producing droplets or aerosols, it is recommended that a Class II Biological Safety Cabinet (BSC) or physical barrier is used (23). As such, all human samples should be treated as potentially infected with any human pathogen, which now includes SARS-CoV-2. This creates a complex matrix in the assessment of potential risk for each sample. Scientific literature and sample history provide us with the information needed to populate this matrix and determine the level of risk presented by such a sample. This matrix feeds into the determination of appropriate controls for assessed samples. Due to the respiratory nature of SARS-CoV-2 transmission, this means we must maintain heightened awareness of all processes that may result in the generation of droplets and aerosols.

A number of factors interplay to determine the final risk associated with running a particular sample on a specific instrument. Effective communication between investigator, shared resource laboratory, and safety officer is critical to ensure a cohesive approach when defining a safety assessment in the context of an SRL (Figure 1). It is recommended that the SRLs, along with their biosafety officer, perform a biological safety assessment for each laboratory group and their specific samples (24, 25). A template for such a risk assessment has been described and reviewed in detail by Schmid, Merlin, and Perfetto (3). In the current time, it is important to pay close attention not only to the types of samples entering shared facilities, where those samples have come from and what risk they might pose, but also the user bringing those samples (Appendix). It is at this point that engineering controls, appropriate PPE and SOPs can start to be applied to control for these risks.

3.1 | Sorters

The ISAC Biosafety committee has written extensively on the assessment of aerosols created by droplet cell sorters and the dangers posed to the sort operator (26). In summary, prior to any cell sorting, a risk assessment needs to be performed that will help identify and mitigate the risks of operator exposure to infectious or potentially infectious aerosols. Once it has been determined that samples can be safely handled through the use of PPE and engineering controls (e.g., aerosol management systems, instruments installed in BSC, etc.), aerosol testing should be carried out to determine if the engineering controls are indeed functioning prior to working with biohazardous samples. The latest published protocol for aerosol testing uses a combination of 1um green fluorescent beads and a relatively inexpensive Cyclex-D aerosol sampling cassette (27). Critical in the aerosol testing procedure is the need to have both a positive control sample (e.g., failure of containment), a normal operation sample, and a sample that follows SOPs in the event of a nozzle clog. This may vary depending on the cell sorter operator, and each SRL needs to establish an SOP, which includes timing for opening the sort chamber door and handling a nozzle after a clog has occurred, to give the aerosol management system time to dissipate lingering aerosols. Each operator should be trained for the SOP prior to performing such a sort, and there may be a need to test each operator for compliance with the SOP, especially in situations where dedicated facility staff are not the only users operating the cell sorter.

Specific to SARS-CoV-2, it has been established by regulatory entities globally that samples containing replication-competent SARS-CoV-2 should be handled in BSL-3 laboratories (28–30). Recently, the ISAC Biosafety committee published an SOP for operation of a droplet cell sorter under BSL3 conditions (31). It is imperative that SRL staff know the source of samples that are coming into the facility. Requiring investigators to fill out pre-sort questionnaires can help the SRL identify sample sources and determine the level of containment required for cell sorting (3).

It should also be noted that a number of microfluidic and chip-based flow cytometry cell sorters have been brought to market in the last 5–10 years. Aerosol generation by these cell sorters is kept to a minimum due to their design; however, there is still a need to validate the sorting safety of these instruments in each environment and with individual users. The ISAC Biosafety Committee has published standards for testing of aerosol management and these standards should be used and adapted to fit each individual situation and instrumentation.

The SRL may decide that only facility staff will operate sorters and room requirements may dictate that only one person can be present. In this context, contact-free sorting can be facilitated by thorough documentation encompassing critical parameters such as the reagents used, the number of sorted cells requested, and suggested gates. Instant communication tools and remote control software (Table 1) are effective for the required interactions such as gate confirmation (8).

3.2 | Analyzers

As discussed above, the operation of cell sorters is well classified due to the significant risk of aerosol generation, with SRL staff trained to ensure safe operation in line with well developed, evidence based SOPs. The use of analyzers is generally considered low risk due to their enclosed systems and low pressures. However, there appears to be little empirical evidence to support this (32, 33). While analyzers can be considered a lower risk than cell sorters, at this time they pose an uncharacterized risk, often operated by a large volume of users with varying levels of experience. As such it is important that strategies are implemented to reduce the risk associated with pathogenic and human samples in the SRL setting (34). These strategies can be subdivided into two main areas: standard operating procedure controls and engineering controls.

3.2.1 | Standard operating procedure controls

Utilizing fixation as a SOP control allows facilities to minimize the risk of running hazardous and potentially hazardous samples in their SRL space. The most common inactivation process utilized for flow

cytometry analysis is the use of formaldehyde solution in various concentrations. Incorporating a fixation protocol into the preparation of samples is a procedure familiar to many users, making this a straightforward process for controlling risk.

Fixation is often performed with the primary goal of stabilizing samples for downstream assays (e.g., intracellular staining). However, fixation protocols designed for stabilization may not necessarily result in pathogen inactivation and special care is needed in the assessment and development of fixation protocols (14, 26, 34–45). Commercial products, both within and across companies, often contain varying concentrations of fixative. This information is often not immediately obvious, and it is therefore necessary to reference the Material Safety Data Sheets (MSDS) along with the protocol when performing fixation protocol assessments. It is important to note that there is inherent variability in the response of pathogen infectivity to inactivation. There is extensive literature detailing pathogen inactivation by varying compounds and this should be reviewed when determining the suitability of a fixation protocol (15, 40, 44, 46–49). We are now seeing literature emerging detailing inactivation of SARS-CoV-2 with formaldehyde solution (16–18, 50), this is summarized in Table 2. In instances where pathogens are emerging or classified as Risk Group 3/4, all fixation and inactivation protocols are recommended to be validated by the laboratory undertaking the research rather than relying solely on literature (28). Viral inactivation validation protocols vary and literature should be reviewed, and local safety officers consulted, when developing protocols for the local context. Viral inactivation validation protocols can be found in these references (15–18, 40, 44, 46–50). In all fixation protocols, it is imperative to consider the: (1) fixative used; (2) how fresh this fixative is; (3) the concentration of the fixative; (4) the time of incubation; and (5) the temperature maintained during incubation (40, 42, 43, 47, 49). Critically, it has been demonstrated in a number of publications that fixation at low temperatures, for example at 4°C, often results in insufficient inactivation of pathogens (42, 43, 47, 49).

It is important that protocols are reviewed and any required changes are identified. Implementing changes in policy can be met with reluctance on the part of the users due to fear of potential impacts on existing work. Facilities can ameliorate this concern by demonstrating that protocol changes do not impact results in any significant manner. Staining protocols, particularly for intracellular markers, may be impacted by additional fixation steps if not implemented with care. Some guidance on staining protocols can be found in this methodology publication (51). Preliminary data from a high-dimensional panel indicate that various fixation protocols do not necessarily alter signal intensity or interpretation of data (Figure 2). Results showed that fixation with a 4% formaldehyde solution (freshly prepared from paraformaldehyde (PFA)) under different incubation conditions did not alter the forward versus side scatter plots (FSC-A vs SSC-A; Figure 2A) or the identification and separation of immune cell populations compared to the unfixed sample (examples of populations can be seen in Figure 2B). Furthermore, the different fixation conditions did not affect the signal intensity of single or tandem fluorophores when the median fluorescence intensity (MFI) of the positive population or the separation between the positive and negative populations (calculated as a separation ratio) was examined (Figures 2C,D respectively).

Significant value lies in testing fixation protocols to determine potential impact on assays. It should be acknowledged that some protocols will not function on fixed samples. The situation may necessitate examination of alternative assays, for example, an apoptosis assay that allows for fixation (52) or a move to implementing engineering controls for such samples. Due to the pandemic, we are now working in an environment with significant inherent risks, so stakeholders will now be seeking out protocols and reagents that facilitate a reduction of this risk. This is an area in which manufacturers have the opportunity to expand their market by identifying new protocols, taking into account viral inactivation and identifying stability of their reagents after fixation.

3.2.2 | Engineering controls

While some engineering controls already exist on instruments, the most effective control for facilities looking to run unfixed hazardous samples may be (as per standard precautions) to enclose an analyzer and any potential aerosols inside a BSC (34, 53). Historically this was not possible due to the size of instrumentation (34), but this is no longer the case with many benchtop analyzers (Figure 3). The ability to enclose a benchtop analyzer in a BSC opens up options for users in the types of protocols and samples that can be run while maintaining biosafety containment. However, a number of factors need to be carefully considered before moving down this path:

1. *Biological safety assessment*: Determine if a BSC is required for the types of samples handled within the facility.
2. *Frequency of live hazardous samples*: Depending on how frequently a facility encounters live hazardous samples, the use of a cell sorter contained within a BSC may be sufficient to accommodate user needs.
3. *Accommodation of instrument within BSC*: Sufficient air flow around instrument within standard BSC for both heat dispersion and maintaining functional containment. Custom BSC options may need to be explored.
4. *Thermal load*: Instrument specifications, such as number of lasers, should be considered. For example, the more lasers, the more heat produced, and the less stable the system may be.
5. *Training*: Adequate training must be provided for appropriate use of the instrument inside the BSC to ensure containment of hazards is maintained.
6. *Accessories*: components such as vortex, pipettes, and tube racks will be needed within the BSC to ensure ease of use and reinforcement of safe behaviors.

Placement of an analyzer inside a BSC increases the burden on facility staff due to the need for additional sample handling training for users and ensuring continued compliance with these behaviors. Additional costs are also associated with the initial BSC purchase and

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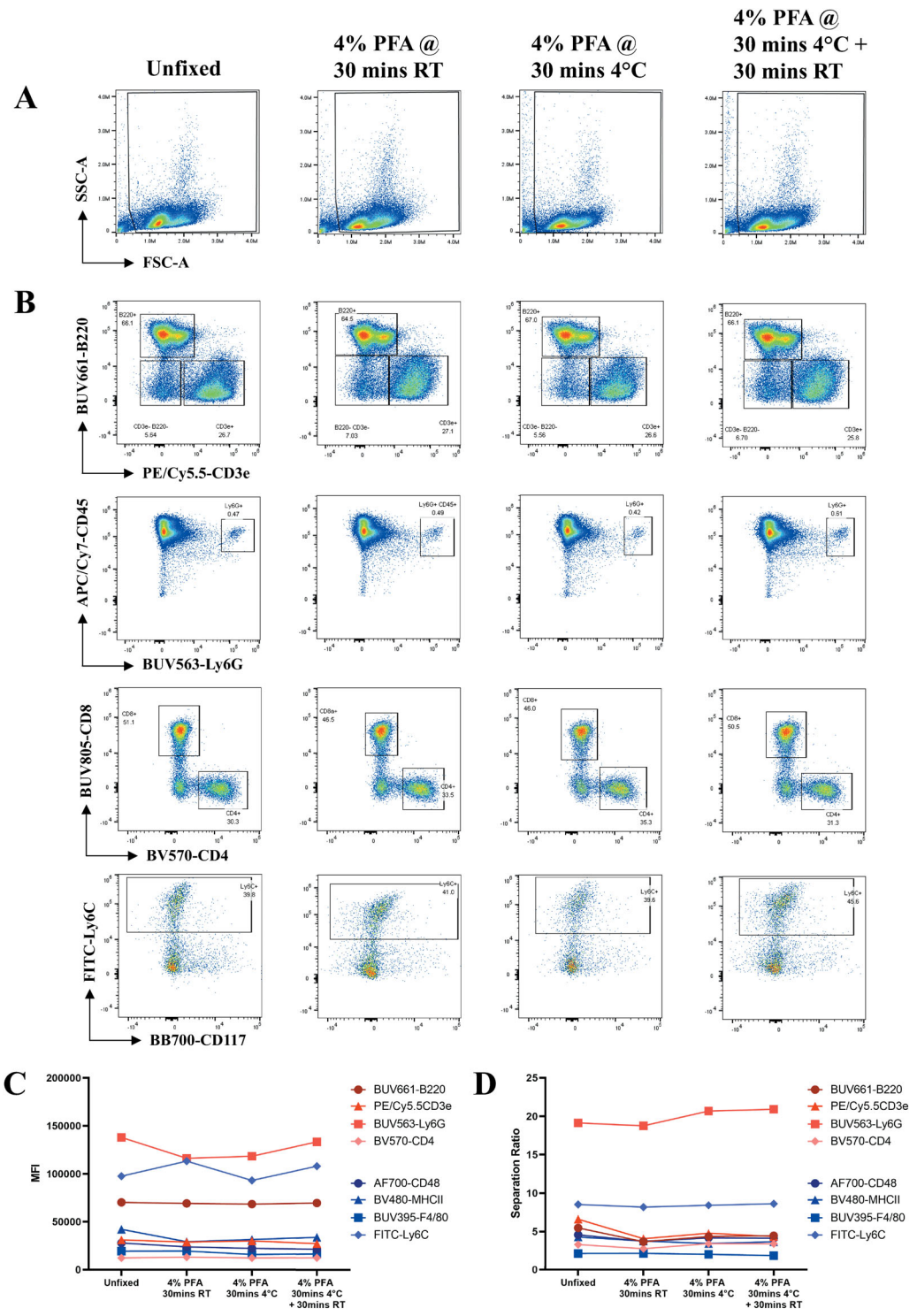

FIGURE 2 Murine spleen cells stained with 25-color high-dimensional panel and treated with four differing fixation protocols: Unfixed, fixed with 4% formaldehyde solution at room temperature for 30 min (4% PFA @ 30 min RT), fixed with 4% formaldehyde solution at 4°C for 30 min (4% PFA @ 30 min 4°C), or fixed with 4% formaldehyde solution at 4°C for 30 min followed by 30 min at room temperature (4% PFA @ 30 min 4°C + 30 min RT). After fixation, cells were washed and immediately acquired on a spectral cytometer, Cytek® Aurora (Cytek® Biosciences, Freemont, CA). The effect of the fixation was examined on (**A**) the forward versus side scatter plots (FSC-A vs SSC-A), (**B**) population identification, separation, and signal resolution of specific immune cell populations, (**C**) the median fluorescence intensity (MFI) of the positive population of single (blue) and tandem (red) fluorophores, and (**D**) the separation ratio between the positive and negative populations of single (blue) and tandem (red) fluorophores. Note: That autofluorescence was not used as a separate parameter for spectral unmixing [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 3 Example placement of a 3-laser benchtop analyzer inside a Class II Biological Safety Cabinet [Color figure can be viewed at wileyonlinelibrary.com]

continued certification. The need for such a set-up is limited and unlikely to be necessary for many SRLs if other measures can be effectively implemented.

3.3 | Instrument Waste

Inactivation of instrument waste is an important consideration for SRLs. Recommendations state that waste containers should hold enough bleach to result in a "10% final concentration of bleach" when the waste tank is full (26, 34). Local regulations and institution guidelines vary considerably and must be considered when developing a protocol for biohazardous waste disposal (54). A number of publications detailing wastewater pathogen inactivation are available and may be used as a guide when developing local protocols (54–59). It should be noted that the stability of bleach is impacted by a number of factors including, but not limited to, pH, temperature, exposure to light, and dilution (59). The management of waste in SRLs should be structured to ensure that waste is exposed to bleach for a sufficient period of time, at an adequate concentration of free chlorine (55). In some situations, such as in BSL-3 laboratory waste streams, autoclaving flow cytometry waste may be considered (26). However, this introduces some complexity due to the potential generation of hazardous gases, such as from wastewater containing formaldehyde or bleach solutions (60).

4 | CONCLUSION

The SRL is a hub for scientific activity, creating a centralized resource that investigators rely on for specialized equipment and technical expertise. The ability to pivot operational structures in response to a pandemic, communicate changed practices, and facilitate continued access has played an essential role not only for research in general, but also in developing our understanding of SARS-CoV-2. Every day we are seeing the emergence of new COVID-19 research, bringing with it potential changes in our understanding and subsequent changes to the safety measures implemented by SRLs. Biological safety assessment needs to consider not only samples and reagents but also the SRL staff, users and visitors as potential risks. Ensuring and maintaining adherence to standard precautions at all times while working within the SRL space will significantly reduce the risk for each individual and subsequently to the wider research community with whom they associate.

The ability of an SRL to rapidly respond to the emergence of a new pathogen centers on having established biological safety assessment procedures in place (3, 25, 34), along with a human risk assessment (Appendix). At this time, literature is starting to form a consensus around the stability and inactivation of SARS-CoV-2 (14, 16–18, 38–40, 43–45, 50). Exactly how these inactivation methods are applied in SRLs will relate directly to the sample type and the level of risk posed. Samples infected with cultured virus should be treated with significant caution, followed by SARS-CoV-2 positive human tissues known to generate propagative virus, and then those tissues not known to carry propagative virus (34, 61–70). Standard precautions apply to all human samples, with a biological safety assessment utilized to help guide the application of additional control measures relative to the local context (1) (Supporting Information Table S1).

Facilitating a safety conversation with users should be the foundation of safety within the SRL. Engaging those who must practice safety measures in the SRL space in these conversations encourages ownership and supports a culture of safety (1). Once a biological safety assessment (Figure 1) and human risk assessment (Appendix) has been completed and measures put in place, it is then the role of the SRL to ensure effective communication, and thus supports users in their ability to comply with these measures. Communication is the key component in ensuring safety during a pandemic.

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Avrill Aspland: Conceptualization; project administration; supervision; writing-original draft; writing-review and editing. **Iyadh Douagi:** Conceptualization; project administration; supervision; writing-original draft; writing-review and editing. **Andrew Filby:** Conceptualization; project administration; supervision; writing-original draft; writing-review and editing. **Evan Jellison:** Conceptualization; project administration; supervision; writing-original draft; writing-review and editing. **Lola Martinez:** Conceptualization; project administration; supervision; writing-original draft; writing-review and editing. **Diana Shinko:** Data curation; formal analysis; visualization. **Adrian Smith:**

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

APPENDIX: EXAMPLE RISK ASSESSMENT FOR POSSIBLE SARS-COV-2 INFECTED USER

This procedure has been developed as an illustrative example to help aid in the assessment of new risks that are now being experienced. Consideration needs to be given to the local context in which this will be applied, including: regional regulation, prevalence of the agent (e.g., SARS-CoV-2), the number of users moving through the space, what measures can be implemented effectively, etc.

Agent Description

Agent: SARS-COV-2
Origin: 2019–2020 pandemic
Susceptible host: Human—all staff, users, and visitors
Disease: COVID-19
Route of transmission: Aerosol, direct contact, fecal-oral, percutaneous
Infectious dose: Unknown
Agent stability: Varies. 3 days up to 28 days depending on surface type
Concentration: Unknown
Work performed at: Physically distant BSL2
Any additional information: Risk assessment assumes that prolonged exposure to infected individuals increases the likelihood of infection. In human populations, infected individuals may shed virus while remaining asymptomatic. Severe risk of illness exists for older adults, people with asthma, or other serious underlying medical conditions (especially those that affect the heart and lungs).

Procedure

Procedures and research goals: Safely open an SRL to provide users adequate access to highly specialized equipment and expert service.
Genetically modified pathogen? No
Containment level for all life cycle stages? All life cycle stages of the agent, including prior to disease onset, may result in high virus shedding. PPE including barriers, gloves, safety glasses, and masks must be used to prevent unintended exposure.
Containment breach: In the event of breach of containment or exposure to an infected individual, the local environment should be immediately cleared of all personnel for aerosol evacuation, followed by surface decontamination. Personnel should be equipped with full PPE as listed above prior to engaging in decontamination.
Pre-treatments and inactivation prior to disposal: Contaminated surfaces must be inactivated with an effective disinfectant for an appropriate length of time, for example, EPA-registered disinfectant active against SARS-CoV-2.
Laboratory testing: Molecular testing is available for SARS-CoV-2 detection in humans.
Environmental disinfection for SRL: EPA-registered disinfectant active against SARS-CoV-2 and accepted by instrument manufacturers will be used. Specifically, any high use areas will be routinely disinfected with 80% Ethanol by wiping with a saturated paper towel and allowing the area to air dry.

Safety Controls

Biosafety level practices: Users entering the facility shall participate in the institution's contact tracing and COVID-19 self-monitoring programs. Users will be allowed to operate instrumentation on an individual basis followed by surface disinfection. All users are required to wear masks and wash hands prior to operating instrumentation. Gloves are highly recommended. Consecutive users shall be separated by a minimum of 15-min intervals to allow aerosols to disperse and limit user–user contact.
Engineering controls: Masks, gloves, safety glasses, and plastic barriers placed between instruments in close proximity.
Clothing: Dedicated laboratory clothing, and dedicated laboratory shoes are recommended.

Personal protective equipment: Approved face mask appropriately fitted, gloves, clean laboratory coat, and safety glasses.

Personnel trained on associated hazards: Mandatory training on how to work with the potentially-infected users is required for all facility staff prior to commencement of research.

Personnel experience: SRL Staff are highly trained to work and adapt in an ever-changing world.

Medical surveillance: SRL staff will monitor incoming users and themselves for symptoms of COVID-19. Symptoms include fever, dry cough, fatigue, and shortness of breath but can also sometimes include headache, aches and pains, sore throat, nasal congestion, runny nose, and loose stool. Identification or self-reporting of any of these symptoms must be conveyed to the appropriate authority.

Region monitoring: Researchers from high risk regions will be asked to transport samples via courier to be run by SRL staff rather than attending the facility in person.

Incident reporting: Anyone experiencing symptoms should alert the laboratory director as well as institutional authority. If infection is confirmed by molecular testing, contact tracing of SRL users for up to 3 days prior to an individual's symptom onset should occur.

Vaccinations: None available

Post-exposure treatment: Contact your medical provider. Infected workers shall quarantine for a recommended period. Exposed individuals should continue to monitor for symptoms and should quarantine as recommended by the local authorities.

Surveillance practices: Users who develop symptoms must immediately report to the laboratory director and institutional authority.

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ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

ISAC INTERNATIONAL SOCIETY FOR ADVANCEMENT OF CYTOMETRY

A Cytometrist's Guide to Coordinating and Performing Effective COVID-19 Research

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Abstract

Cytometry is playing a crucial role in addressing the COVID-19 pandemic. In this commentary—written by a variety of stakeholders in the cytometry, immunology, and infectious disease communities—we review cytometry's role in the COVID-19 response and discuss workflow issues critical to planning and executing effective research in this emerging field. We discuss sample procurement and processing, biosafety, technology options, data sharing, and the translation of research findings into clinical environments. © 2020 International Society for Advancement of Cytometry

Key terms

SARS-CoV2; COVID-19; pandemic; flow cytometry; immune monitoring

The world is immersed in a global health challenge on a scale not seen since the 1918 influenza pandemic. COVID-19, caused by the novel SARS-CoV2 virus, has led to nearly 600,000 reported deaths worldwide (as of mid to July 2020) (1) and crippled economies. In the midst of this public health emergency, many researchers and laboratories have shifted focus toward the study of SARS-CoV2. Presently, many aspects of SARS-CoV2 infection are not fully understood, but there are active research efforts underway studying viral transmission and immune responses, as well as international efforts to develop vaccines and therapies.

The human body is made up of 37 trillion cells, which can be grouped in cell populations based on shared phenotypic identities or functional specialization. For the past several decades, cytometrists have been busy dissecting the heterogeneity of these cellular populations (2), and in the process revealing targets of viral infection, identifying protective immune cells, and characterizing the immune responses that lead to protection or to tissue damage. These are critical needs for the world's COVID-19 response, so understandably cytometry is now playing a crucial role in SARS-Cov2 research. As cytometric technologies are more widely implemented in this new research setting, workflow questions surrounding technology choice, biosafety protocols, quality control, and data sharing are emerging. Moreover, as cytometry is deployed for immune monitoring in vaccine and drug trials, it must be integrated into large studies, some of which are occurring across hospitals taxed by the hefty workloads of their local epidemic, with limited time and resources for planning or preparation; these issues present important challenges as well.

In this commentary, we—as stakeholders from various cytometry-associated disciplines—highlight the value of cytometry in the study of COVID-19, and chart a path for how

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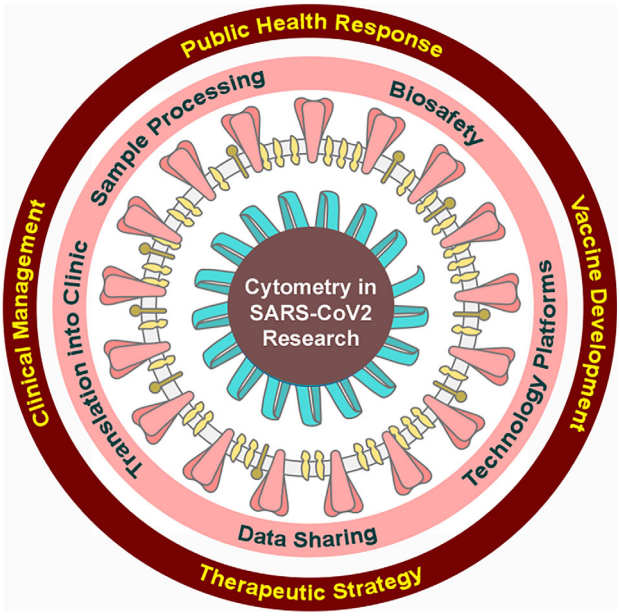

Figure 1. Considerations in COVID-19 research are listed in the pink ring, and broad areas of relevance are described in the red ring. [Color figure can be viewed at wileyonlinelibrary.com]

cytometry can be applied and translated successfully to address this grave public health threat (Figure 1).

Cytometry's Current and Future Role in Combating COVID-19

Cytometry, in all its various forms and with all its associated disciplines, has already built the foundation of basic research in COVID-19 immunology (3). Within months of the declaration of a worldwide pandemic, our community has defined immunologic abnormalities in patients, found associations between immune characteristics and disease severity, and begun to characterize the T-cell response to virus. The quick response of cytometrists to COVID-19 research opportunities is not surprising; the early stages of COVID-19 research have involved the "plug and play" of our existing toolbox into a new disease setting.

For a novel disease like COVID-19, the identification and enumeration of different cell types are the foundation upon which further discoveries, and candidate interventions, are built. Early phenotypic analysis of immune cells in COVID patients showed that lymphopenia, and in particular a loss of T-cells in circulation, was a hallmark of disease (4). Using high parameter flow cytometry, one study classified COVID-19 patients into three groups, based on the immunophenotypic state of their T-cell compartment: those patients with CD4+ T-cell activation similar to that observed in acute infections (but only moderate CD8+ T-cell activation), those with highly differentiated CD8+ T-cells, and patients with little indication of immune activation or perturbation. Notably, individuals with the first immunophenotype (CD4+ T-cell activation) tended to have more severe disease (5). Other studies have identified the loss of suppressive myeloid cells (6), characterized eosinophils as expanding in the first week (7), and revealed an increase in plasma-blast B-cells (8) as features of COVID-disease that may be associated with disease severity.

Beyond broad phenotypic and functional analysis, cytometry is also used for the critical task of analyzing and evaluating immune responses to viral components. A patient's immune response to specific viral components can be assessed by measuring functional outcomes such as proliferation, cytotoxicity, activation, cytokine secretion, or antibody production upon re-exposure to viral antigens in vitro, or through the direct analysis of cells that bind antigen. The latter is possible using specialized fluorochrome-tagged reagents called "peptide–MHC multimers," for the analysis of viral antigen-specific T-cells, and antigen trimers for similar analysis of B-cells. These tools will play a central role in the evaluation of vaccine candidates, as they are developed. Most cytometry-related technologies are capable of analyzing both bulk and antigen-specific immune responses with single-cell resolution.

In the first peer-reviewed results from a COVID-19 vaccine-trial, the NIH/Moderna mRNA1273 vaccine (9) was shown to generate SARS-CoV2-specific CD4+ T-cells that expressed the TH1 cytokines IFNg, IL2, and TNF, but not the TH2 cytokines IL4 and IL13. Notably, past work in mouse models of coronavirus infection showed that immune-mediated pathology was associated with TH2-type responses, and TH1- and CD8-biased responses in the animal model were associated with better outcomes (10, 11). Early COVID-19 research has also demonstrated an important role for IL17-producing T-cells in disease (12), suggesting that anti-IL17 blocking monoclonal antibodies (already available for use in other diseases) might have therapeutic benefit in COVID-19 patients (13). Studies have also shown that antigen-specific T-cell responses are common in convalescent patients (CD4+ and CD8+ responses against SARS-Cov2 are observed in 100% and 70% of recovered patients), and suggest cross-reactive immunity (since up to 60% of *unexposed* patients have detectable SARS-CoV2-specific CD4+ T-cells) (14). These studies only represent a few of the myriad of studies pre-published on bioRxiv and medRxiv to date, many of which demonstrate the clear value of single cell, high parameter flow cytometry for patient- and animal model-oriented COVID-19 research.

Flow cytometry also offers a unique ability to purify certain cell subsets—or antigen-specific cells—for downstream analysis using fluorescence-activated cell sorting (FACS). FACS is a critical step for the development of one class of COVID-19 therapeutics, known as passive immunotherapy. In these early phases of the pandemic, passive immunotherapy for COVID-19 has largely involved the infusion of convalescent patient plasma, containing SARS-CoV2 neutralizing antibodies, into severely ill patients. Using FACS, it is possible to identify the B-cells making SARS-CoV2 antibodies, sort them into single-cell wells, clone them, and then characterize the neutralization ability of antibodies produced by each

SARS-CoV2-specific B-cell. The B-cells that produce neutralizing antibodies can thereby be distinguished from those making non-neutralizing antibodies (which do not prevent infection), and sequenced. Using this approach, the sequence of B-cell receptor (i.e., antibody) genes that make neutralizing antibodies are revealed, and these sequences can be transferred to genetically engineered cell lines for the mass production of neutralizing antibodies, which can then be administered to patients (15). Mass produced, neutralizing antibody therapeutics (developed using cytometric technology) are the next phase of passive immunotherapies, and represent a potentially important disease treatment strategy with advantages over convalescent plasma.

Sample Quality, Procurement, and Processing

Given the rapid pace of COVID-19 research, and the growing number of laboratories providing immune monitoring for COVID-19 studies, there is an urgent need to harmonize sample collection across sites, and share information about sample stability and marker integrity with sample storage. It is incumbent upon COVID-19 researchers to carefully define procedures and variables in their studies and to adhere to published standards and guidelines for sample processing and analysis. These steps will ensure that the field moves forward quickly, with robust and meaningful data.

In particular, sample quality presents a major challenge in the context of a rapidly unfolding pandemic. It is well known that variables such as collection tube, sample age, and storage conditions can affect cell viability, deplete some cell subsets, alter protein/transcript expression, or the ability to respond in a functional assay (16, 17). These factors may introduce experimental artifacts that affect data reliability, so understanding the impact of sample quality is critical and must be considered when interpreting data. However, with the rapid pace of SARS-CoV2 research, and the reality of performing research work in the context of a busy clinical setting, extensive evaluation of the impact of preanalytical variables in advance may not be feasible. In these situations, which are not ideal, information from peer reviewed publications, guidelines, and standards, can provide the basis for defining criteria for sample rejection. It is beyond the scope of this communication to make specific recommendations, as the requirements for each individual assay will be different, but immunophenotyping assays provide an example of the need for very specific guidelines. In immunophenotyping, the impact of viability may depend on specimen type, storage conditions, and stability of the cellular population assessed (18). To further complicate matters, some immunophenotyping assays are performed in a whole blood matrix that includes mild proprietary fixation chemicals, which are designed to extend specimen stability. In this format, viability measurements are not possible. In contrast, immune function tests by ICS or ELISpot assays (which are commonly used to evaluate vaccine efficacy), often use a common threshold of >66% viable cells (19,20). In any case, clear standards for sample rejection should be employed not only for settings governed by Good Clinical Lab Practice and regulatory guidelines but should also be seriously considered for nonregulated and nonclinical settings (21,22).

A unique challenge in COVID-19 research arises from delays in the release of patient samples, while SARS-CoV2 testing is performed. This situation presents a major challenge, since the sample's condition may be questionable by the time it is received by the flow cytometry laboratory. Each investigator must weigh the risk/benefit of testing precious COVID-19 patient samples, which may be of suboptimal condition, but if tested, a sample's condition should always be recorded. If a full understanding of the impact of preanalytical variables is not known at the time of testing, investigators are advised to conduct thorough preanalytical evaluation at a later date in order to establish acceptance criteria for sample quality (23). If this is done, the previously tested samples which would not have met the acceptance criteria can be flagged and removed from the final data set.

A second challenge arises from the lymphopenia observed in COVID-19 patients. Immunophenotyping assays must be selected and designed to account for the low number of lymphocytes present in patient peripheral blood. FACS-based purification of rare antigen-specific cells for downstream immunoassays, which would typically be possible from 10 million healthy donor cells, may require two to three times more sample from COVID-19 patients. A key step in experimental design will therefore be to assess the limit of detection and sensitivity of any assay used for COVID-19 research. Similarly, the screening of samples for antigen-specific T-cell populations may be uniquely challenging in COVID-19 patients. Investigators may need to employ highly multiplexed approaches (24), such as barcoding peptide–MHC Class I multimers with unique combinations of dyes, in order to acquire more information from a single, low yield sample.

The challenges described above highlight the critical need for efficiently and clearly sharing methodological information within the COVID-19 scientific community in "real-time" rather than relying on standard peer-reviewed publication timelines. In an effort to meet this need, several journals, including *Cytometry Part A*, have adopted a process for expedited peer review. In order to have the most impact, it is especially important that the methods sections in the fast-tracked publications are complete and include details of specimen collection and processing. Additionally, professional societies can facilitate the exchange of methodological information, using web-based resources like those proposed at the conclusion of this article.

Biosafety

Because SARS-CoV2 is a novel pathogen, most institutions performing COVID-19 research have had to perform risk assessments and develop biosafety strategies unusually quickly. The ability to perform COVID-19 research is highly dependent on this process; in fact, in the United Kingdom, ethical approval for research is not granted without evidence that biosafety protocols are in place. In the United States,

local institutional biosafety committees (IBCs) typically perform risk assessments independently of Institutional Review Boards (IRBs), but still biosafety approval is a critical step in the research process.

At the time of publication, in the United States, research using SARS-CoV2-infected blood must be performed under Biosafety Level 2 (BSL2) conditions, with enhanced precautions, at most institutions. These so-called "BSL2+" (or BSL2/3) precautions include personal protective equipment (gloves, face mask, splash resistant gowns, eye protection, and head/shoe coverings), a system for positively disinfecting waste, as well as capped tubes and centrifuge buckets (opened only within a biosafety cabinet). The rationale for working with blood under these conditions (which are less strict than BSL3 requirements) stems from research showing that the blood is not a significant source of infectious virus. Although viral RNA can be detected in blood, to date, no viable virus has been recovered in the small studies that have been performed, and there have been no cases of laboratory-acquired disease amongst people working with blood from SARS-CoV2 patients or those infected with the agent responsible for 2003's South Asian SARS epidemic. Moreover, even transfusion of blood products containing SARS-CoV2 RNA has not resulted in infection. BSL3 precautions are required, however, when propagating virus, working with tissues (25,26) where active replication is present (such as BAL and lung), or when there is significant risk of exposure to aerosols containing infectious particles.

Cell sorting instruments and their varied potential to generate aerosols, represent a good example of why biosafety policies have to be developed based on local risk assessments. Classical droplet-based sorters vary widely in their potential for aerosol generation, and the protocols to measure these aerosols may or may not be implemented locally. At some institutions, droplet-generating sorters are encased in biological safety cabinets, reducing the risk of aerosol exposure. At other institutions, aerosol-free microfluidic sorters are available for COVID-19 research to replace droplet sorters.

In summary, biosafety policies for COVID-19 research should be developed at the institutional level, under the guidance of local safety experts, based on the kinds of samples, equipment, and workflows that will be used for a project. Moreover, these policies should be revisited frequently; SARS-CoV2 is a novel agent, and our understanding of it is subject to change. Resources are available to guide this process (27-29), including those developed by the International Society for the Advancement of Cytometry's (ISAC) Biosafety Committee. ISAC's biosafety guidelines discuss broad principles (relevant for all pathogens) and also provide means for testing containment and monitoring/training staff.

Cytometry Technologies Available to Study COVID-19

In recent years, cytometry has grown well beyond its roots in fluorescence-based cell analysis. Technologies in the cytometry arsenal now include high-parameter fluorescence flow cytometry, spectral flow cytometry, mass cytometry, molecular cytometry, single-cell RNA-sequencing, imaging flow cytometry, imaging mass cytometry, Isolight Single Cell Cytokine Secretion, CODEX, and spatial transcriptomics. In broad terms, these technologies can be grouped into those that measure mRNA transcripts versus protein (or both), or alternatively into technologies that query single-cell suspensions versus those that examine tissues. These groupings are helpful in evaluating which technologies are best suited for a particular COVID-19 study.

Solely in terms of the sheer volume of data provided, technologies that measure mRNA transcripts are unparalleled. In their most targeted form, these technologies—using for example BD Bioscience's Rhapsody system and Precise Assays—measure 400 predefined transcripts simultaneously from each cell in a sample. In the broadest implementation of mRNA analysis, the transcriptome can be measured (using various instrumentation and protocols), providing a broad characterization of cells. It is important to recognize the limitations of mRNA-based platforms, however. First, gene expression is inherently noisy, occurring in bursts and with long silent phases. Moreover, post-transcriptional regulation often governs the final expression of a protein, or its stability on the cell surface. These biological phenomena conspire to affect the correlation between mRNA and protein. Thus, a lack of mRNA expression does not necessarily mean the protein is absent; nor does the presence of an mRNA target prove expression of a protein. Second, sensitivity is heavily dependent on the depth of sequencing, which is impacted by the cost-considerations, the expression levels of abundant and rare transcripts, and the number of cells examined. Single-cell sequencing data are typically sparse, with many cell-gene combinations absent. Third, these technologies are very low throughput and expensive. They are not well suited to quickly characterizing rare SARS-CoV2-specific T-cells, for example, and are too expensive and labor-intensive to deploy for large studies of COVID-patients, (especially for studies that aim to collect samples for every patient admitted to an emergency room, e.g.). The latter consideration is critical. COVID-19 is a disease with great heterogeneity, in terms of the severity of the disease, the age of patients, and the presence of absence of comorbidities. Powered appropriately, studies will be large and samples must be analyzed quickly, on-demand, as they arrive. Single-cell RNA sequencing is not well suited to such settings. Molecular cytometry, a related technology that replaces antibody fluorescent tags with oligonucleotide tags, overcomes some limitations of unimodal single cell RNA sequencing by directly measuring protein expression. However, the technique requires capture and isolation of single cells from a sample, using a single cell capture device (like the 10X Chromium or BD Rhapsody systems). These capture devices are limited to the capture of no more than 20,000 cells per capture, which is very low for immunophenotyping rare cell populations (but certainly sufficient to study immune landscapes); the analysis of more cells is certainly possible, but the need for additional materials increases cost and labor.

In contrast, single-cell analysis of cellular proteins typically provides a good combination of throughput, cost, and data content (30). Technologies based on the measurement of cellular proteins include high-parameter flow cytometry, spectral flow cytometry, mass cytometry, imaging flow cytometry, imaging mass cytometry, and CODEX. These technologies all use antibodies to mark cellular proteins; the antibodies carry different tags (e.g., fluorescent dyes, elemental isotopes) based on the method of detection used for each platform. In particular, high-parameter flow, mass, and spectral cytometry can support the analysis of 1 million cells or more from each cell sample, and patient samples can be analyzed on automated platforms. The samples used for these studies are often stable for longer than those used for mRNA-based studies, since generally proteins are less labile than mRNA transcripts, and less affected by changes in cellular environment. The primary keys to success when using these technologies are authentication/titration of antibodies (31) and standardization of instrumentation across sites (32) (which often presents a considerable challenge). A major limitation of these technologies is their relative complexity. For flow and spectral cytometry, the design of antibody panels is critical and time consuming; while for mass cytometry, instrument calibration, as well as reagent production and qualification can require considerable effort. In these regards, sharing of antibody panels across the COVID-19 cytometry community may be very helpful (33), as would the creation of local "libraries" of antibody reagents.

Cytometry technologies may also be grouped by the matrix in which the measurements are made. Technologies that measure cells in suspension include: flow, mass, spectral, imaging flow, and molecular cytometry, along with IsoLight's single-cell cytokine secretion platform and single cell RNA sequencing. Technologies that measure cells within tissue include imaging mass cytometry, CODEX, and spatial transcriptomics. In the most general terms, tissue-based technologies will have little value for the study of patients with asymptomatic or mild disease, because these conditions are not accompanied by tissue pathology. Moreover, ethical approval is not likely to be granted to sample a lymph node or lung tissue in relatively healthy individuals. Blood is much more accessible and available for these patients, so studies that involve healthy or early stage individuals will likely use technologies that measure cells in suspension. Yet, it is important to remember that severe COVID-19 disease typically manifests in lung tissue, so deploying tissue-based technologies is likely to provide the biggest experimental value at a lower cost in understanding severe disease. Where tissue is not available, but interventional pulmonologists are accessible, an alternate approach for studying tissue-resident immune cells is to perform bronchoalveolar lavages (BAL), which "wash" cells out of lungs for collection.

When deciding what approach is best for analyzing COVID19 samples, it is important to appreciate the inverse correlation between parameters and throughput. Fluorescence flow cytometry is still by far the most cost-effective approach to single cell analysis, but it is limited by parameter space. As such, lower parameter flow cytometry may be seen as a technology used to confirm findings. At the other end of the scale, molecular cytometry provides the parameter space to perform very deep and comprehensive analysis of single cells. It is more likely to discover new and unappreciated heterogeneity, but it is generally not well suited to large studies, because cost considerations preclude scalability. The reality is that a combinatorial approach of very high parameter/low throughput "discovery" and focused, lower parameter/higher throughput confirmation will be the best approach. Studies will likely use mostly suspension cytometry technologies, because successful translation into a clinical setting will require more standardized approaches in which the key questions/parameters are distilled down to what is minimally required to call cell phenotype or function.

Particular cytometry technologies allow unique applications that could be of great value to COVID-19 projects. In a previous section, we described how fluorescence flow cytometry offers live cell sorting, which plays a key role in the development of passive immunotherapy using neutralizing antibodies. Similarly, immunoassays that characterize cell functions like proliferation are uniquely performed in fluorescence flow cytometry (and likely spectral flow cytometry) settings. The beauty of these approaches is that other measurements can be multiplexed in a single step providing a deep, simultaneous characterization of cell phenotype and function. As described above, from a discovery perspective, we expect that molecular cytometry technology, with its unmatched information content, will yield great insight into SARS-CoV2 pathogenesis. Using this technology, it is also possible to examine the clonality of T-cells and B-cells in COVID-19 patient samples, by sequencing T-cell receptor and immunoglobulin genes, respectively. This approach represents a powerful means to map the immune response to COVID-19 antigens, a key element of vaccine studies. Finally, imaging flow cytometry technologies capture not only the average fluorescence of a marker bound to a cell but also the specific location of that signal on the cell. Currently available systems can measure up to 12 parameters per cell; however, since the output is based on digital imagery, it is possible to derive a near limitless number of parameters that often require advanced analysis approaches such as deep learning to extract meaning. In any case, this technology will likely provide information about where on the cell surface SARS-CoV2 particles are bound.

Data Sharing

The efficient response of the scientific community to this pandemic will require the sharing of not only knowledge but also the data from which that knowledge is derived. This is especially important for data arising from technologies with high information content, like multidimensional cytometry. With the rapid pace of COVID-19 research, it is unlikely that a laboratory generating a data set can completely mine their own data before publication, so opportunities to further extract knowledge are missed when data are not freely distributed in

the community. A fine example of both the use and distribution of publicly available data sets is illustrated by the work of a Belgian research group (34), who provided an immune atlas of BAL specimens from patients with mild COVID-, severe COVID-, or non-COVID-related pneumonia. Data from non-COVID-related pneumonia were re-analyzed from their previous study, providing important context for their COVID-related findings. Similarly, a data set comparing healthy children and those with Kawasaki syndrome (a multisystem inflammatory disease) to the COVID-related manifestation of this syndrome has recently been posted to medRxiv (35). This data set will be remarkably valuable because these syndromes are quite rare, and the COVID-related manifestation was only very recently reported.

Publicly available flow cytometry data from COVID-19 research are also being used in new and unique ways. A key challenge in flow cytometry centers around the discrimination of cells expressing a marker versus those that lack expression ("gating"). Gating can vary significantly between data analysts, and automated gating algorithms do not always capture the gates that human analysts would have set. This discordance can have downstream effects, making it harder to interpret comparisons between patient groups because some cell populations are over- or under-represented in the dataset. This challenge may be solved by crowd-sourcing gating across a wide range of analysts, as a new project is currently testing (36). This project "gamifies" publicly available flow cytometry datasets, allowing anyone—including the lay public—the opportunity to set their own gates as part of a web-based video game.

Translation of Research Findings into Clinical Environments

The objective of translational and clinical science has always been to accelerate the bench-to-bedside progress. This objective has never been more urgently needed as during the COVID-19 pandemic. Much of the effort in SARS-CoV2/COVID-19 research is directed toward the identification of biomarkers that predict control or progression of disease, or assess novel therapeutic agents and strategies. Promising findings will need to be evaluated in clinical laboratories, for their potential to be incorporated into clinical care. In addition, there will be a strong push to develop the most interesting findings into diagnostic tests. These efforts will require laboratories to meet regulatory guidelines, which may be reviewed more quickly by regulators, but are unlikely to be substantially loosened or ignored. As such, the generation of high quality, well-documented, quality-controlled data from clinical laboratories and manufacturers is paramount.

For successful translation of research findings to clinical environments, the process of generating robust data must begin in research settings and is accomplished by adhering to previously established criteria when performing research assays. These guidelines include: (1) the Minimal Information About T-cell Assays (MIATA) project (37); (2) the International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (38); (3) documentation describing Good Clinical Laboratory Practice (39); (4) the Clinical Laboratory and Standards Institute (CLSI) guideline H62: Validation of Assays Performed by Flow Cytometry (40). These guidelines supplement good flow cytometry practice (29) and present a framework for reporting flow cytometry data and qualification of flow cytometry assays in a manner that provides detailed documentation and is most consistent with the regulatory requirements for final approval of an assay or reagent for clinical use. An important example lies in data generated to support FDA-approval of vaccine trials, where data from multicolor flow cytometry assays of T-cell phenotype and function is often used to document the immunogenicity, or immune correlates of protection, for a vaccine candidate (41). There is a great deal of historic experience to draw from in this area, as validated flow cytometry panels have been in use in HIV vaccinology and immune monitoring for some time. Data to meet regulatory requirements, and indeed the antibody panels themselves, are likely to be easily ported to the SARS-CoV2 settings by connecting the right researchers.

The key elements for successful translational and clinical science involve standardization (42), harmonization, and method validation. As we are still in the early days of SARS-CoV2 research, progress in standardization and harmonization is critically needed. For example, descriptions of antigen-specific T-cell responses to SARS-CoV2 have differed across studies because of the varying coverage of peptide pools. An important step forward will be to standardize reagents in the field, particularly peptide pools. Similarly, to enroll the number of patients required for studies of COVID-19 pathogenesis or vaccine immunogenicity, multiple centers will be required. To compare results across multiple trial sites in a reliable and structured manner, external quality assurance programs, like those implemented for HIV/AIDS vaccine trials will be needed (43). Facilitating multicenter translational studies, antibody reagent mixtures dried in single test tubes can be produced on demand by major companies. Apart from driving assay standardization at the reagent level, the use of dried/lyophilized antibody panels for flow cytometry will also help in simplifying the experimental workflow and relieving the burden on hospital and research staff conducting critical research while dealing with a local epidemic. In sum, efforts to harmonize work from multiple SARS-CoV2 laboratories will greatly benefit the public health response to COVID-19.

Conclusion

Professional societies that support cytometry can play an important role in the scientific response to COVID-19. This May, the International Society for the Advancement of Cytometry (ISAC) formed its COVID-19 Workgroup (https://isac-net.org/page/COVID-19). The group acts as a focal point, bringing together scientists worldwide working on SARS-CoV2, or otherwise interested in the role of cytometry technologies and methodologies in the pandemic response. The group includes significant representation from the

International Clinical Cytometry Society (ICCS) as well, because the COVID-19 work performed in research settings will need to be translated rapidly into clinical laboratory settings.

Given the magnitude and severity of the pandemic, people across diverse disciplines—in research, industry, and clinical laboratories—have shifted their focus to SARS-CoV2. The breadth and depth of science represented will surely benefit society, but the greatest benefits can only be realized if expertise is efficiently shared amongst scientists. Already, as addressed here, there are common questions emerging about how to plan for SARS-CoV2 research, including issues of biosafety, sample procurement, experimental design, and data analysis. The ISAC COVID-19 Workgroup will assist in these areas, providing a centralized source for biosafety guidelines, a forum to discuss experimental design, a networking directory to establish collaborations, and a connection to data repositories and analysis algorithms. In today's climate, with the unfortunate politicization of science, it is more important than ever that laboratories generate high quality, reproducible data with traceable, calibrated measurements; ISAC's COVID-19 Workgroup will help shape that process.

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Conflicts of Interest

The authors have no conflicts of interest to report.

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SRL COMMUNICATION

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Risk awareness during operation of analytical flow cytometers and implications throughout the COVID-19 pandemic

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Abstract

The COVID-19 pandemic has brought biosafety to the forefront of many life sciences. The outbreak has compelled research institutions to re-evaluate biosafety practices and potential at-risk areas within research laboratories and more specifically within Shared Resource Laboratories (SRLs). In flow cytometry facilities, biological safety assessment encompasses known hazards based on the biological sample and associated risk group, as well as potential or unknown hazards, such as aerosol generation and instrument "failure modes." Cell sorting procedures undergo clearly defined biological safety assessments and adhere to well-established biosafety guidelines that help to protect SRL staff and users against aerosol exposure. Conversely, benchtop analyzers are considered low risk due to their low sample pressure and enclosed fluidic systems, although there is little empirical evidence to support this assumption of low risk. To investigate this, we evaluated several regions on analyzers using the Cyclex-d microsphere assay, a recently established method for cell sorter aerosol containment testing. We found that aerosol and/or droplet hazards were detected on all benchtop analyzers predominantly during operation in "failure modes." These results indicate that benchtop analytical cytometers present a more complicated set of risks than are commonly appreciated.

KEYWORDS
aerosols, biosafety guidelines, Core Laboratory, COVID-19, flow cytometry, pandemic, risk assessment, SARS-CoV-2, Shared Resource Laboratory, SRL

1 | INTRODUCTION

The emergence of SARS-CoV-2 led to rapid implementation of multiple control measures to help mitigate its spread, including wearing face coverings and monitoring for signs of fever. Since then, the research community has built a foundation of literature on SARS-CoV-2 transmission, stability, and inactivation that can be used to develop biological safety assessments [1–7]. Updated laboratory policies have been implemented to address the risk of person-to-person viral spread. These policies build on "Standard Precautions," developed in response to the HIV/AIDS epidemic of the 1980s [8,9]. Standard precautions include hand washing, wearing appropriate PPE, and using respiratory hygiene, while handling any human specimen regardless of infection status. If there is risk of aerosolization or droplet hazards, additional physical barrier controls are implemented [10]. The generation of aerosols is an established risk factor for laboratory acquired infections [11–15] and has been linked to the transmission of pathogens not usually known to transmit via this route [11,16]. Ultimately, SARS-CoV-2 has generated heightened scrutiny of biosafety considerations, especially for instrumentation. In particular,

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analytical cytometers have been underrepresented in risk assessments, and their potential hazards are not well documented.

Biological safety assessments are invaluable tools for flow cytometry facilities. They facilitate communication between the principal investigator, SRL staff, and safety officer, ensuring that all parties are aware of experimental risks. Considerations examined by these assessments must incorporate hazards associated with the biological samples and any known infectious agents, as well as the instruments on which samples will be manipulated, tested, or processed. The outcome of biological safety assessments is the application of controls to ensure personnel safety: Engineering, PPE, SOPs, and Administrative/Leadership [17]. It is important to note that, when applied to analyzers, the information used to formulate the biological safety assessment may be well known for some variables while relatively unknown for others. Because of this, the controls put in place as an outcome of biological safety assessments may contain deficiencies.

Guidelines for aerosol risk management during cell sorting were first published in 1997 [18] and have been incrementally refined with the latest general guidelines published in 2014 [19]. COVID-19 specific recommendations have also recently been added [20]. Today, evidence-based biosafety guidelines for cell sorting are widely accepted. Biosafety committees can factor this in-depth understanding of aerosol risk related to cell sorting into facility-specific control measures. As a result, flow cytometry facilities are able to manage and mitigate potential risks during cell sorting.

Known risks have contributed to standardized guidelines for cell sorters; however, the assumption that benchtop analyzers are low risk has resulted in variability across different facilities in how analyzers are handled during biosafety risk assessments. This presumption of low risk is informed by two factors: (1) analyzers operate under lower sample pressure, which reduces aerosol risk and (2) the fluidics system is considered completely enclosed compared to the open stream of cell sorters. So far there has been little empirical evidence to support this statement of low risk, and the COVID-19 outbreak has served as an additional incentive for SRLs and other labs to reassess analyzer biosafety risk [21,22]. Operating an analytical cytometer involves routinely handling potentially biohazardous components, such as the instrument waste tank or the automated plate loader (APL) and sample injection port (SIP), when running samples in configurations where the fluidics system is not fully enclosed. If these regions produce biohazardous material, then the instrument may facilitate pathogen transmission. In this study, we evaluated a broad range of analytical cytometers under various operating conditions to identify potential biosafety hazards at key test locations. Ultimately, this article is intended to increase user awareness of potential risks associated with analytical cytometers.

2 | MATERIALS AND METHODS

2.1 | Instrument test area

Seven distinct instrument models from six separate vendors, across four SRL sites, all routinely serviced by their respective manufacturers. These were evaluated using the Cyclex-d/Dragon Green bead assay as developed for cell sorters to identify aerosol and/or droplet generation [23]. The instruments represent a range of fluidic designs ensuring that evaluation of test areas would be widely applicable. Not only do fluidic designs differ between instrument models, but in some cases, variability within the same model was also noted across sites. Each test area was assessed under "normal operating conditions," which describe an analyzer that has not been modified beyond manufacturer specifications for biosafety considerations. Throughout testing "failure modes" were identified, these incorporate manufacturer design specifications that appear to be deficient in aerosol containment (i.e., vent holes in waste tank with no filter) as well as inadequate containment that is commonly encountered as a result of instrument deficiencies. Instrument deficiencies include clogged fluidic lines, expired aerosol filters, or circumventing safety features such as removing backflush containment on the SIP and running with no enclosure on the APL.

Each instrument model was evaluated at two separate SRLs, and one instrument was tested in three locations. Although the physical design and layout of each instrument varied, aerosol sampling locations were applied consistently. Four distinctive test areas were identified using a risk assessment based on the fluidics system and general instrument design. It was anticipated that particle escape might occur due to certain mechanisms or fluidic design features along the sample path, beginning with sample injection and terminating with waste collection. These four test areas are depicted in Figure 1.

2.2 | Air sampling

Air sampling was performed as previously described [23]. In brief, aerosolized fluorescent 1.0 μm Dragon Green (DG) beads (Excitation [Ex] 480 nm; Emission [Em] 520 nm; Bangs Laboratories, Fishers, IN) or PBS background control were collected within a Cyclex-d impactor cassette with an internal coverslip used for particle detection (Environmental Monitoring Systems, Charleston, SC). The cassette was connected to a MegaLite vacuum pump and Rotameter (Environmental Monitoring Systems, Charleston, SC) so that consistent and repeatable vacuum was produced. The vacuum pump was set at a constant 20 L/min.

A 1:100 dilution of DG beads was utilized for all test areas. Each tested instrument had slightly different hardware and corresponding mechanisms for controlling fluid movement through the cytometer. As a result, steps like acquiring, mixing, and rinsing samples for single tubes or 96-well plates vary slightly from instrument to instrument.

2.2.1 | Sample injection port

Some instruments pressurize the sample tube, while others use a syringe mechanism or peristaltic pump to draw up sample volume. For SIP assessment, the air sampling cassette was positioned 5 cm from bottom of SIP during the intervening SIP backflush. A tube of DG beads was loaded for 3–5 s in acquisition mode and unloaded five times.

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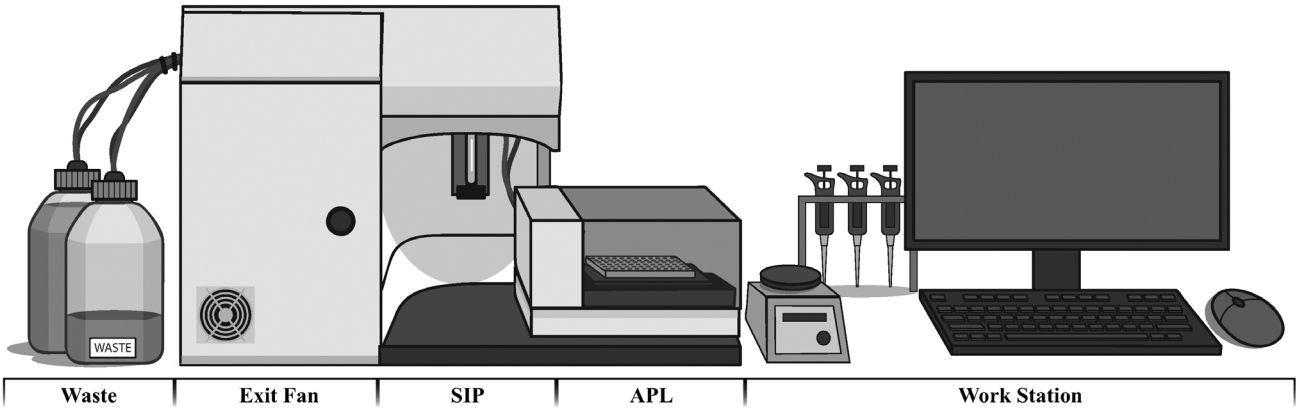

FIGURE 1 Standard benchtop analyzer workspace with associated equipment, including the following test area locations: Waste tank, exit fans, sample injection port (SIP), automated plate loader (APL), and workstation

2.2.2 | Automated plate loader

To address whether or not APLs generate aerosols during acquisition, DG bead sampling was performed both inside and outside of the plate cover (or instrument chassis). Regardless of instrument type and method for aspiration, mixing, and backflush, standard software APL settings were utilized with an acquisition stop time of 1 s/well. Half of a 96-well plate, unless otherwise stated, was run on instruments with the vacuum impactor placed roughly 5 cm away from the measurement site to capture potential aerosol and/or droplets generated during acquisition [23].

2.2.3 | Exit fans

Fans were tested at either the main instrument body or at separate APL consoles, if present. Air sampling cassette was placed 5 cm from exit fans during plate acquisition as described previously.

2.2.4 | Waste tank

The waste tank was tested under three conditions: enclosed, waste tank filter off, waste tank cap off. This testing did not apply to every tank due to individual design differences. All air sampling conditions were measured during plate acquisition as described previously. The "enclosed" condition represents the highest level of containment achievable for a given tank design. For this condition, all fittings and caps were closed and filters were placed into vent holes in tanks, where present. For measuring the enclosed condition the air sampling cassette was placed 5 cm from the waste tank filter if present, otherwise this was placed 5 cm above the waste tank cap. The instruments with vent holes were tested without filters for the "filters off" condition in a similar manner with air sampling cassette 5 cm from the open vent hole. The "waste tank cap off" condition was used for determining the worst case scenario for this test area. For measuring the open waste tank, the sampling cassette was placed 5 cm directly above the waste tank opening.

2.3 | Droplet containment failure

To simulate droplet containment or backflush failure during tube acquisition, the waste line on the droplet containment module was pinched off causing sample/sheath backdrip. A tube containing 1 ml of 1:100 DG beads was run on "low" flow rate for 10 s. The tube was removed and five droplets were collected from the SIP in consecutive 1.5 ml eppendorf tubes from 1 to 25 droplets, approximately 120 s. These aliquots were counted using a Denovix® Celldrop™ FL cell counter, and the number of DG beads per five droplets was calculated.

2.4 | Bead enumeration with fluorescence microscopy

After air sampling, coverslips from within the Cyclex-d cassettes were removed and placed on gridded microscope slides. Each collaborating institution developed their own workflow for DG microscopic enumeration using respective positive and negative controls to ensure accuracy. Table 1 shows the microscopes and methods used for DG counting at each site. Three of four sites utilized manual counting, while one site utilized a high-content bioimaging system and a semi-automated approach for enumeration.

3 | RESULTS

3.1 | Identification of test areas associated with analytical flow cytometers

Four regions of biological concern, or test areas, were identified and evaluated for potential aerosol and/or droplet hazards. As noted previously, these test areas include the sample injection port (SIP), automated plate loader (APL), exit fans, and waste tank. In total, seven instrument models from six vendors were evaluated. Data detailing aerosol generation from these test areas along with corresponding

TABLE 1 After air sampling, coverslips were transferred to slides for microscopic enumeration. The microscope, magnification, optical filter, and summary of DG bead enumeration methods for each of the four collaborating sites is displayed

Site	Scope	Magnification	Filter	Bead enumeration method
1	Axio Scan.Z1 (Carl Zeiss, Göttingen, Germany)	10×	500–550nm	Manual counting using positive control events to set illumination intensity for verifying positive events
2	Labophot (Nikon, Tokyo, Japan)	10×	BA520	
3	EVOS® (Thermo Fisher Scientific, Waltham, MA)	10×	525/50	Image analysis performed using PerkinElmer Harmony 4.8 software
4	Opera Phenix™ (PerkinElmer, Waltham, MA)	5×	500–550nm	The PhenoLOGIC™ machine learning module was used to identify and quantify the number of fluorescent particles per coverslip

controls are summarized in Table 2. Raw data can be found in Supporting Information Table S1. Most test areas were further evaluated to determine efficacy of containment measures under adverse circumstances or "failure modes," which highlight the broader scope of biosafety risks associated with these regions and with benchtop analyzers in general.

3.2 | Sample injection port

The SIP for single tube acquisition was the first area tested. Six of seven available instruments are capable of running single tubes, while the seventh instrument is designed to acquire samples from plates only. Under normal operating conditions, all instruments, except Instrument A, have enclosed fluidic designs to limit aerosol and/or droplet hazards. Fourteen of 15 tests showed no DG bead counts above background level detection under normal conditions, even though the SIP encounters a high concentration of potentially hazardous material due to sample loading and acquisition. Instrument A is the only instrument with an open or unenclosed backflush, and subsequently it displayed an elevated bead count.

The SIP is also a site of common "failure modes." Although few DG beads were detected under normal conditions, there is a significant aerosol and/or droplet risk when sample backflush or rinsing is compromised. The highest number of beads was found in the first five drops with a 71% reduction by drops 5–10, 96% reduction 11–15, 97% reduction 16–20, and 99% reduction by the final 21–25 drops, as demonstrated by Figure 2.

Another common "failure mode" is dripping or spraying while backflushing due to misalignment between the backflush and the aspiration port. This particular 'failure mode' was tested only on one instrument, and a significant number of DG beads (>100) were detected there (data not shown).

3.3 | Automated plate loader

All seven instrument models tested were equipped with an APL; however, specific APL layouts varied dramatically from instrument to instrument. Some APL systems, along with all moving parts, were entirely enclosed within the main instrument chassis, while others were external to the main instrument. Under normal operating conditions, most devices are equipped with a plate/chamber cover. Although a considerable variability between instruments was observed, DG bead counts were relatively high within the APL chamber (see Table 2). This observation was somewhat expected, since all APL systems were equipped with both sample mixing and probe washing stations, which are likely sources of aerosols. Five instruments used an uptake and expulsion method for mixing, whereas two instruments (D) used a vortexing method. We also expanded testing to outside the APL plate cover, since DG beads were detected inside the plate chamber and APL covers are not sealed against aerosol escape. We detected fewer DG beads outside the APL cover compared to the number we captured within it. This finding seems to suggest that operating with the plate/chamber covered may partially contain some aerosols generated by the APL.

A "failure mode" on Instrument C (site 3) was also noticed and corrected during aerosol testing. Of note, it was the measured elevation of aerosols that instigated the investigation into the potential instrument defects. Eventually it was determined that a partially clogged waste line had produced the increase in DG bead counts (data not shown).

3.4 | Exit fan

All flow cytometers are equipped with fans responsible for cooling electronic components. Some of these fans are located within the vicinity of fluidic components, so that if aerosols are being generated, exit fans will likely disperse them. If electronics and exit fans were completely separate from fluidics, data was not collected. For most instruments, exit fans did not show any noticeable DG beads; however, instrument A (sites 1 and 4), which had higher bead counts in the sample backflush and APL chamber regions, also had higher bead counts at the exhaust fan than any other instrument.

3.5 | Waste tank

The final test area was the waste tank. Waste tank design varied greatly between instruments and vendors, and many instrument models have multiple options for sheath and waste management. Despite the variability in design, waste tanks were separated into three

TABLE 2 Results of Cyclex-d/DG bead testing across instrumentation, sites, and test areas [Color table can be viewed at wileyonlinelibrary.com]

Instrument	Location	Average Background	Sample Introduction Port	Automated Plate Loader		Exit Fan	Waste Tank			Positive Control
			Backflush	Inside	Outside		Enclosed	Filter Off	Cap Off	Manual Aerosol
A	1	1	21	25	20	15	21	>100	>100	>100
	2	0	0.3	0.7	0	0	1.3	5.3	>100	>100
	4	1	1.5	>100	9.5	12.5	ND	>100	>100	>100
B	1	1.5	2.5	10	4.5	NA	4	3	ND	>100
	2	0	0	0	0	NA	0	34.3	>100	>100
C*	3	0	0.67	10.3	0.3	NA	0.3	NA	>100	>100
	4	3.75	ND	9.75	1.5	NA	0.5	NA	>100	>100
D	3	0	0.67	2.3	0	0	0.3	27	>100	>100
	4	1	3	2	3	1	3	50.5	>100	>100
E	3	0	0	0	0	0	0.3	50	ND	>100
	1	3	10	2	2	5	NA	27	NA	>100
F	1	1	0	2	1	2	15	NA	ND	>100
	2	0	0	13.3	0	0.4	0.7	ND	69.3	>100
G	3	0	NA	2.3	ND	0	NA	ND	ND	>100
	2	0	NA	0	ND	0.7	NA	ND	ND	>100

[a]For all 96-well plate samples only 1 row acquired per repeat

Average Number of Dragon Beads

0 50 100

Notes: Counts represent the average number of beads per slide.
Abbreviations: NA: Not applicable, ND: Not done.

categories: waste tanks fully enclosed with a filtered vented cap, waste tanks with open vented holes, and waste tanks with the cap removed. While an entirely sealed tank would theoretically pose the lowest biohazard risk, many tanks are installed with vent holes that do not have filters in place. A selection of instrument waste tanks used during testing as well as some highlighting "failure modes" are shown in Figure 3.

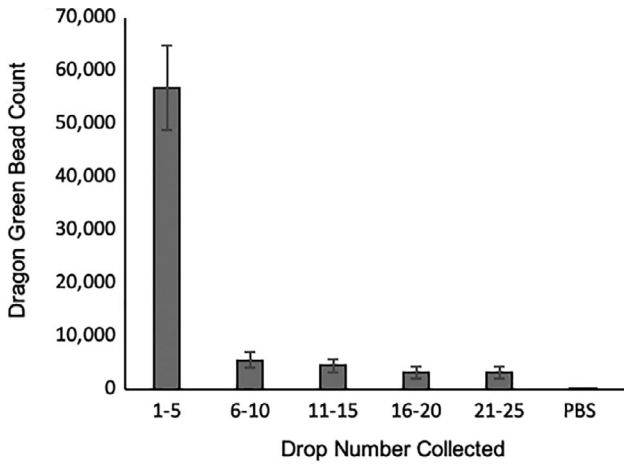

FIGURE 2 Droplet containment failure results. Five droplets were pooled in each sample with a total of 25 droplets collected. After collection, DG bead counts were enumerated for each pooled sample. Experiment was repeated five times with replicate bead count per five drops displayed, including standard deviation as shown

The waste tank had the highest rate of aerosol generation, per detection with the Cyclex-d assay (see Table 2). All instruments tested displayed a higher number of DG beads with a capless waste tank. For most instruments, a filter reduced the bead counts compared to the waste tank without a 0.2 μm filter in place. An increase in DG beads released through waste vent holes was evident on five of the seven instruments, with Instrument A (site 1) recording results above 100 (Table 2). These results indicate that a high number of aerosols were being generated inside the waste container (>100 DG beads), with some escaping through the vent hole.

4 | DISCUSSION

Benchtop analytical cytometers present a unique set of biosafety issues, many of which require additional scrutiny by SRLs. The majority of analytical services provided by SRLs are "self-run," meaning that users generally have reduced interaction with SRL staff. The volume

FIGURE 3 Waste tank caps. All instruments except bottom right have clear vent holes. Top row demonstrates instruments lacking air filters within vent holes. Bottom row demonstrates instruments with air filters placed within vent holes [Color figure can be viewed at wileyonlinelibrary.com]

of projects, investigators, users, and hours for analyzers can also make oversight difficult; this is compounded by limited staffing and resources. This challenge has been amplified by the pandemic, leaving many users to manage instrument "failures" with reduced in-person support. This situation can result in unsafe operating conditions if the user is unable to identify and appropriately respond to instrument "failure modes." Most importantly, current SRL policies and safety guidelines rely on the conventional knowledge that analyzers are low risk, thus affording them little attention beyond the generation of individual SOPs.

SRL safety guidelines for analyzers typically focus on user compliance with SOPs under normal conditions without assessing safety considerations during "failure modes." In contrast, when SRLs assess safety and readiness for cell sorting, they simulate a "failure mode," mimicking the common aerosol-generating event of a clogged or partially clogged nozzle. Aerosol testing during and after this "failure mode" allows SRLs to develop policies protecting sort operators under worst-case operating conditions. For example, the time needed for an Aerosol Management System (AMS) to evacuate the sort chamber can be incorporated into an SOP as the amount of the time to wait before removing the nozzle and opening the sort chamber for cleaning after a clog. SRLs are also responsible for validating that the AMS is operational; the operational status of the instrument and its safety features are not assumed based upon manufacturer installation or guidelines. Similarly, analyzer biological safety assessments must consider instrument function under both normal and "failure modes," regardless of manufacturer certification. Normal conditions can only occur after a thorough evaluation of the instrument and after any identified deficiencies are rectified.

To date there are few publications that directly evaluate the safety of benchtop analytical cytometers. One recent publication performed an assay looking at the growth of specific bacteria after air sampling at three locations within a flow cytometry facility: preparation workbench, at the cytometer between the operator and the SIP, and at an additional bench within the facility [21]. This publication did not seek to identify whether aerosols are produced, but rather sought to assess the risk of running their specific bacteria live on a benchtop analytical cytometer. The results of this publication found that none of the three specific bacteria could be grown after air sampling, suggesting that either minimal or no aerosols were produced, or the bacteria did not survive the flow cytometry procedure and thus did not grow after air sampling. We have begun to address this question further by adapting the Cyclex-d/DG bead assay to evaluate a range of potential areas of risk along the instruments' fluidic systems.

Across all sites, instruments returned positive results from the Cyclex-d/DG bead assay, most markedly at the waste tank and as a result of failures. However, this assay, as previously published, has been developed and validated specifically for cell sorters, which are high-pressure instruments. The concentration and size of aerosols generated by cell sorters are well characterized, enabling SRLs to identify their potential for significant risk to operators [23]. Characterization of this assay, as applied to benchtop analyzers, has not been completed, so aerosol and/or droplet concentration and size, along with the frequency of DG bead-containing aerosols, remains unknown. Taken together, our results strongly suggest that benchtop analytical cytometers do generate aerosols and/or droplets, predominantly at the waste tank or during instrument "failure modes." This is an important consideration when undertaking biological safety assessments and applying safety controls.

Nearly all detected "failure modes" originated from clogged fluidic lines or waste tank deficiencies. Of note, "failure modes" were identified on every instrument model tested. For example, each instrument had its own unique "failure mode" resulting in a dripping SIP, which poses an aerosol and/or droplet hazard. Dripping could be caused by many factors, including a bent sample probe, clogged tubing, faulty vacuum/pumps, or instrument design featuring an open dripping backflush. Interestingly, there were no observable differences between pressurized and vacuum driven aspiration in this study. Differences were more likely to be attributed to nonenclosed fluidic paths such as backflushes, APL aspiration and mixing, and open vent holes on waste tanks. In some instances, individual instrumentation results varied between sites. The exact causes for this have not been investigated due to the size of the study. It is likely that unidentified failure during operation, instrumentation configuration variability, limits of detection of DG bead enumeration between sites, or environmental laboratory factors such as ventilation and air circulation may have all contributed to this variation. Future studies including a larger number of laboratories are important to allow a broader assessment across sites.

Ventilation, air circulation, and droplet dynamics are an important consideration for laboratories, with small droplets and evaporated droplet nuclei remaining suspended in air [24]. In the context of flow cytometry, these droplet dynamics are of significant importance in the risk assessment of instrumentation and samples. Droplet dynamics in cell sorting has long been a topic of discussion, with efforts leading to the [25] characterizing aerosol generation during partial blockages on a FACS Aria II cell sorter. In the absence of appropriate containment, these aerosols form droplet nuclei that have little likelihood of settling out of the air [12,24]. Techniques to reduce the aerosol burden in the SRL space should help to reduce the risk posed by aerosol generating procedures. Such techniques include: reducing the number of individuals using the space, reducing user overlap, using appropriate PPE and increasing ventilation and air circulation in the lab area.

Waste tanks were a major source of aerosol and/or droplets across all instrumentation tested. Some instrument vendors seemed readily aware of this hazard, with preinstalled clearly labeled biosafety filters on the waste container. However, many tested instruments did not include this safety feature. In fact, multiple instruments had waste tanks with vent holes for air release without a containment measure applied, such as a 0.2 μm filter. This variation in waste tank configuration can only be identified and addressed if the instrument undergoes a risk assessment prior to use. In a larger sense, the general waste tank variability also highlights the unaddressed risk associated with analytical cytometers. The risk posed by the waste tank is significant, this risk is particularly present during any procedure requiring the removal of the cap, such as during waste disposal. While measures should be in place to ensure the inactivation of waste prior to disposal, additional safety measures such as appropriate PPE use should be considered when developing SOPs for waste tank handling and waste disposal.

While not tested here, many workspace accessories pose additional aerosol and/or droplet generation risk. It is well established that the use of pipettes and vortexes generate aerosols and/or droplets [26], both of which are common benchtop analyzer accessories. This, combined with the results obtained here, demonstrate the need to treat workspace surfaces with additional care with regard to biohazard exposure. While instrument and surface decontamination should already be incorporated into benchtop analyzer SOPs, this should serve as a reminder of its importance in the safety of SRL spaces. Another aspect that acts to reduce the risk posed by aerosols and/or droplets is sample preparation. Wherever hazardous or potentially hazardous samples are to be run on benchtop analyzers, steps should be taken to inactivate potential pathogens. Inactivation of pathogens for flow cytometry can be achieved through the implementation of an appropriate fixation protocol, if this is not possible, samples should be run on an instrument contained within a BSC [27].

The SRL Services Committee of the International Society for the Advancement of Cytometry (ISAC) sent out a survey to assess how facilities were adapting to the COVID-19 pandemic [28]. Of the 123 responding laboratories 68% never fully closed at any point, of the 32% of laboratories that did close, only 11% were still shut down in mid-May of 2020. These statistics highlight the essential services provided by SRLs within the global research community. The ability to maintain, or return to, operations is centered on having strong biological safety assessment, risk assessment, and SOP processes in place. This study demonstrates detection of aerosol and/or droplet hazards on all benchtop analyzers tested, indicating that there is a level of risk associated with the operation of these instruments. SRLs are encouraged to revisit and update SOPs within the local context with a focus on reducing this risk. We have developed lessons learned below focused on ensuring safety during benchtop analyzer operation. Further studies are required to characterize the specific level of risk associated with benchtop analyzers, this will then facilitate the development of best practices documentation.

5 | LESSONS LEARNED

As noted in the cell sorter best practices document [29], every SRL is responsible for making their own informed decisions and weighing risk factors within their specific context to develop a comprehensive biosafety-related analyzer policy. To assist SRLs and other laboratories in addressing the risks associated with benchtop analyzers, we have developed some specific advice. It is recommended that a risk assessment, relative to the local context, be performed to help guide in the application of the below additional recommendations:

- Monitoring: Qualified SRL staff should perform a thorough inspection of all instrument test areas to identify any regions of concern, paying particular attention to potential hazards such as sample line blockages, leaks, and integrity of waste tank barriers. After initial identification and resolution, instrumentation should be inspected routinely.
- Biological safety assessment: Performing a detailed biological safety assessment for each project taking into account both the

experimental procedure, the nature of the analyte and the specific analytical instrument involved will help in guiding the choice of control measures. Control measures for potentially hazardous samples should include fixation for inactivation, or containment within a BSC. This process is described in detail by Aspland et al [27].

- Training: It may be beneficial to educate instrument users on how to identify and respond to potential failures to ensure containment of hazardous aerosols and/or droplets. Training during a pandemic is discussed in more detail by Daniels et al [30].
- Sample introduction port: Dripping, priming, and backflush mechanisms may facilitate the spread of hazards and the opportunity for contamination in this region is high. It is recommended that all practical measures are taken to reduce this hazard via the use of appropriate hand hygiene, glove use and additional cleaning of the workspace. For example, do not bypass the droplet containment module on SIP by removing the outer sleeve of the sample probe or continued operation with an obstructed waste line.
- Automated plate loader: Operation of the APL should be performed as per manufacturer instructions with the use of a barrier control, such as the APL enclosure. Failure to do so may result in exposure to aerosol and/or droplets.
- Exit fans: Most fan locations are such that they do not generate air turbulence over sample handling regions. Heightened scrutiny should be applied to any instrument with a fan operating over sample handling regions.
- Waste tank: The use of barrier controls on waste tanks is common but varies across instruments and locations. Care should be taken to inspect waste tanks for potential aerosol and/or droplet escape, particularly via misplaced caps, vent holes and damage. All vent holes should be considered a region of concern and a filter installed as a barrier control. Advice should be sought from the instrument and filter manufacturers as to what type of filter may be suitable. The use of any filter requires careful monitoring to ensure integrity and instrument function as aged or wet filters are no longer effective controls and may impact instrument function. Further to this, waste should be appropriately inactivated [27] prior to disposal and the use of appropriate PPE during waste tank handling and disposal should be considered.
- Workspace: SRLs should place greater emphasis on PPE compliance during analyzer use in anticipation of "failure mode" incidents. Standard precautions include wearing gloves, gowns, masks and protective eyewear or face shields, and hand washing. This, along with standardized workspace cleaning procedures should be implemented to limit the risk associated with contaminated surfaces.
- Ventilation and air circulation: Such techniques include limiting the number of individuals using the space, reducing user overlap, using marks, and increasing ventilation and air circulation.

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AUTHOR CONTRIBUTIONS

Avrill Aspland: Writing-original draft; writing-review and editing. **Claude Chew:** Writing-original draft; writing-review and editing. **Iyadh Douagi:** Writing-original draft; writing-review and editing. **Tessa Galland:** Writing-original draft; writing-review and editing. **Michael Solga:** Writing-original draft; writing-review and editing. **James Marvin:** Writing-original draft; writing-review and editing. **Josh Monts:** Writing-original draft; writing-review and editing. **Dayton Nance:** Writing-original draft; writing-review and editing. **Adrian Smith:** Writing-original draft; writing-review and editing.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

DATA AVAILABILITY

Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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ISAC CYTOMETRY
Journal of Quantitative Cell Science PART A

Handling and Processing of Blood Specimens from Patients with COVID-19 for Safe Studies on Cell Phenotype and Cytokine Storm

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The pandemic caused by severe acute respiratory syndrome coronavirus 2 heavily involves all those working in a laboratory. Samples from known infected patients or donors who are considered healthy can arrive, and a colleague might be asymptomatic but able to transmit the virus. Working in a clinical laboratory is posing several safety challenges. Few years ago, International Society for Advancement of Cytometry published guidelines to safely analyze and sort human samples that were revised in these days. We describe the procedures that we have been following since the first patient appeared in Italy, which have only slightly modified our standard one, being all human samples associated with risks. © 2020 International Society for Advancement of Cytometry

• **Key terms**
SARS-CoV-2; Covid-19; coronavirus; biosafety; cytokines; cytometry

THE dramatic epidemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes Corona Virus Disease-2019 (Covid-19), started in China in late 2019 and has rapidly spread worldwide (1). In Italy, the first patients with severe pneumonia were observed in Lombardy, and the first confirmed case dated February 21, 2020. The exponentially growing number of infected people can now be traced in a website that is continuously updated (2).

As of the end of March 2020, the city of Modena is dealing with >1,500 cases and about one-fifth are hospitalized. We have been deeply involved in monitoring the immune system of patients at different stages of the disease, including those asymptomatic, taking novel therapies, requiring intensive care. The analysis that was requested was related to lymphocyte phenotype along with a few functional assays to identify skewing toward T helper type 1 (TH1) or T helper type 2 (TH2) differentiation. The purpose of this report is to provide the first experience of the Modena Covid-19 Working Group (MoCo19) on handling, processing and analyzing by flow cytometry blood specimens obtained from patients with Covid-19. Here we describe our procedures in studying peripheral blood mononuclear cells (PBMCs) isolated from infected samples with the intent to provide indications for performing relatively simple immunological studies and reassure the flow cytometry community, currently on the frontline to the fight against the virus (3), since there are no particular risks if all precautions are taken.

Laboratory Biosafety

Risk Assessment

We first conducted a local risk assessment to address safety or security risks. At this level, risks were defined and characterized, and mitigation measures were implemented accordingly. All laboratory processes, including locations, procedures, and equipment used, were discussed and defined by the risk assessment team of our University. Since panic is often the first reaction of those who are not in the lab but work in the same area, it is strongly recommended to contact and reassure them and the administration personnel explaining how the safety procedure are respected when starting studies on this topic.

SARS-CoV-2 belongs to the Coronaviridae family and is taxonomically related to the subgenus Sarbecovirus (4). This is an enveloped virus containing a single-stranded positive-sense RNA as viral genome. Virions are spherical, with the spiked glycoprotein embedded in the envelope. Additional viral proteins include envelope, matrix, and nucleocapsid. The presence of SARS-CoV-2 RNA across different specimens, that is, bronchoalveolar lavage fluid, fibrobronchoscope brush biopsy, sputum, nasal swabs, pharyngeal swabs, feces, blood, and urine, has been quantified by real-time transcriptase-polymerase chain reaction (RT-PCR) (5). According to these first data, a small percentage (1%) of blood specimens had positive PCR test results for viral RNA. Moreover, the median PCR cycle threshold value reported was 34.6 (range: 34.1–35.4, 95% confidence interval [CI]: 0.0–36.4) suggesting that a low concentration of viral RNA is present in the blood.

In principle, finding viral RNA in a fluid does not mean that RNA has the original length, nor that it works. No solid information is currently available regarding the detection of infective SARS-CoV-2 particles in the blood, nor on the real meaning of viral RNA present in plasma. In fact, the natural route of transmission is person-to-person, and there are no reports of laboratory infections. In fact, the infection occurs primarily via direct contact or through droplets spread by coughing or sneezing from infected individuals (6). However, a recent study reports that the viral load in nasal and throat swabs from an asymptomatic patient was similar to that of symptomatic patients, indicating that infected persons with no symptoms can transmit the virus, likely with the same infectivity (7). Regarding the stability of SARS-CoV-2 in aerosols and on various surfaces, it has been reported that the virus can remain viable and infectious in aerosols for hours, and on surfaces up to days (depending on the inoculum shed) (8). Even if finding viral RNA dos not mean finding an infectious virus, this suggests that surfaces must be accurately cleaned with hypochlorite and ethanol.

This provides the first rule: at work, including during breaks, lab meetings, and data discussions, if individuals are in close proximity or just if more than one person is present in the same room, everybody must always wear a simple surgical mask (not a Filtering Face Piece Type-2 [FFP2] mask, which does not filter exhaled air). Unlike disposable gloves, surgical masks can be used several times along multiple days.

Laboratory Working Areas

Any handling, processing, and testing of blood specimens from Covid-19 patients need to be performed in appropriately equipped laboratories by competent personnel, previously trained on the technical and safety procedures. National guidelines on the laboratory biosafety should be followed in all circumstances, and general information is also available in the World Health Organization (WHO) Laboratory Biosafety Manual (9). In Modena, blood specimens from patients with Covid-19 are handled in Biosafety Level (BLS)-2 laboratory supplied with Class II biological safety cabinets (BSC). All cabinets are daily equipped with an internal waste (containing 0.5% bleach) where any possible contaminated biological material is discarded.

All Laboratory workers must wear personal protective equipment. In details, when working in the laboratory area, personnel need to mandatory wear disposable gloves, laboratory coat, and surgical mask, required to prevent the spread of unwanted droplets. This precaution is also important to prevent the infection spreading in case a researcher is asymptomatically infected. Laboratory clothing is maintained in the lab and should never be used outside. Laboratory doors are kept closed during all experiments in progress.

A distance of at least 1m is maintained between people inside the lab and, if possible, the presence in each room should be limited to one person only. If not possible, it is important not to have two operators using the same instrument (e.g., like a cabinet 180 cm large), nor two researchers sitting too close in front of the same flow cytometer or of the same computer.

Manipulation of Blood and Analysis and PBMCs from Covid-19 Patients

Packaging and Transport

Blood specimens from confirmed cases, collected by adequately protected and trained physicians at the patients' bed are transported to and between laboratories as UN3373, "Biological Substance, category B," and are placed in two secondary containers to minimize the potential for breakage. Opening of containers is performed inside a certified Class II BSC in a manner that reduces the risk of exposure to an unintended sample release.

Handling and Processing

During specimen manipulation in a Class II BSC, personnel wear two pairs of disposable gloves, laboratory coats, surgical mask, and eye protection. The use of two pairs of gloves is mandatory to work in BSC, so that at the end of the procedure the external layer of gloves is removed and discarded into the waste located inside the BSC. FFP-2 masks are also available and are used for personnel protection during specific procedures, including cell sorting or stimulation/activation of living cells. It is better to perform these procedures alone, and

thus this type of mask can be used—only when operating and not close to other people.

According to the WHO Laboratory Biosafety Manual, for procedures with a high likelihood to generate aerosols or droplets (e.g., vortexing, mixing, sonication or centrifugation), a certified Class II Type A1 or A2 BSC should be used. During the procedure for the isolation of PBMCs from peripheral blood, centrifugation steps are at high risk to generate fine-particulate aerosols and droplets. However, centrifuge buckets are sealed for centrifugation, and specimens are centrifuged in securely capped polypropylene tubes that are loaded and unloaded in a Class II BSC. As additional precaution, every step of the procedure is performed in a Class II BSC to minimize the risk of exposure to an unintentional sample release. Only disposable plasticware and pipettes are used, which are decontaminated into the internal waste.

On completion of work, the internal waste is closed and discarded into a biosafety waste. Surfaces are decontaminated typically with 0.5% bleach and then with 70% ethanol.

Handling of PBMCs for Phenotype Analysis

To date, no data are available regarding the ability of SARS-CoV-2 to infect PBMCs. If SARS-CoV-2 behaved like all respiratory viruses, the blood from Covid-19 patients should not contain infective particles. However, waiting for definitive reports, and according to standard precautions, we prefer to take into consideration the fact that in principle plasma and mononuclear cells obtained from blood may contain transmissible infectious agents, and must be handled in a Class II BSC. Indeed, this is what we have been doing for many years when analyzing human blood from patients with different physiological conditions (for example, age, from 0 to 110 years, or pregnancy) that is always treated as if it were infected with a pathological agent like human immunodeficiency virus (HIV) or hepatitis B virus. So, many years ago—I would say more than 30—we started to strictly follow first safety procedures, then the indications given by the International Society for Advancement of Cytometry (ISAC) (10,11), with the recent updates (see: https://isac-net.org/page/Biosafety).

As we well know, for the analysis of cell phenotype by flow cytometry sample preparation typically includes isolation of PMBCs, staining with monoclonal antibodies (mAbs), incubation for a short period, washing, and fixation. Then, fixed samples are acquired by using an instrument that, in our case, is located in a locked BLS-2 room. Personnel involved in sample preparation handle PBMCs specimens in a Class II BSC and wear laboratory coat, gloves, surgical mask, and eye protection.

As additional precaution, even if not required, those involved in sample acquisition can even wear FFP2 instead of surgical mask. After the acquisition, the flow cytometer is washed for 15 min with 0.5% bleach, 15 min with cleaning solution and finally 15 min with deionized water. At the end of the acquisition, the entire working area is cleaned-up by using disinfectant solution (1/10 volume dilution of 0.71 M sodium hypochlorite, then 70% ethanol). Disposable materials (collection tubes, gloves, pipettes, tips) are discarded into appropriate biohazard containers with hypochlorite and all work surfaces are wiped off.

A potential exposure to infectious materials, or any sort of accident has to be immediately reported to the head of the laboratory for the appropriate evaluation. Needless to say, activities like eating, drinking, smoking, handling of contact lens, applying cosmetics, playing with the phone or chatting on social networks are absolutely prohibited. In Table 1, the

Table 1. Summary of the personal protective equipment and collective protective devices for handling and processing blood specimens and PBMCs from Covid-19 patients

PROCEDURE	PERSONAL PROTECTIVE EQUIPMENT	COLLECTIVE PROTECTIVE DEVICES
Handling of blood	• Surgical mask • Two pairs of gloves (the external to be used only when working in the BSC) • Eye protection • Lab coat	• Class II BSC in a BLS-2 lab
Staining of PBMCs	• Surgical mask • Two pairs of gloves (the external to be used only when working in the BSC) • Eye protection • Lab coat	• Class II BSC in a BLS-2 lab
Acquisition at the flow cytometer (fixed cells)	• Surgical mask • Gloves • Eye protection • Lab coat	• BLS-2 lab
Acquisition of unfixed cells: requires cell sorting procedures	See https://isac-net.org/page/Biosafety	• BLS-3 lab

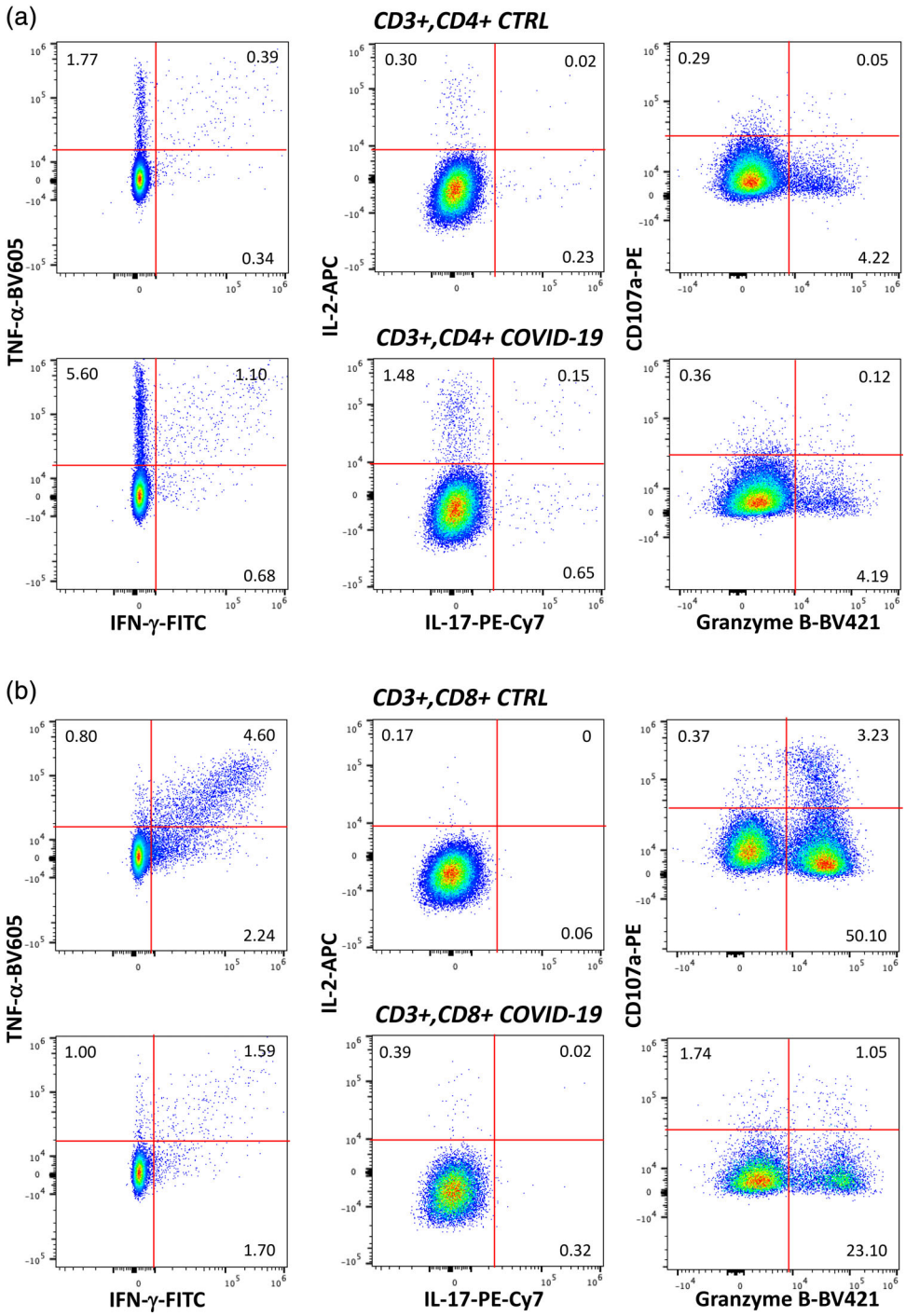

Figure 1. Representative example of cytokine production by CD4+ and CD8+ T cells from a Covid-19 patient with severe pneumonia after in vitro stimulation after in vitro stimulation with anti-CD3/CD28 (1ug/mL) for 16 h in the presence of anti-CD107a-PE (Biolegend, San Diego, CA).(14,15) PBMC were stained with viability marker (AQUA Live Dead, ThermoFisher) and anti-CD4-AF700 and CD8-APC-Cy7 (Biolegend). Cells were fixed and permeabilized with Cytofix/Cytoperm (Becton Dickinson, San Josè, CA) according to manufacturer protocols. Finally, cells were stained with anti-IFN-γ-FITC, anti-TNF-α-BV605, anti-IL-17A-PE-Cy7, anti-IL-2-APC, and anti-Granzyme B-BV421 (all from Biolegend). Data were acquired by using attune NxT acoustic flow cytometer. (A) Intracellular staining of different cytokines in previously gated living CD3+,CD4 + in a healthy donor (upper plots) and in a patient (lower panels); (B) intracellular staining of different cytokines in previously gated living CD3 +,CD8+ in a healthy donor (upper plots) and in a patient (lower panels); (C) analysis of the polyfunctionality of CD8+ T cells by using "Simplified Presentation of Incredibly Complex Experiments (SPICE),", kindly provided by Dr. Mario Roederer (NIH, Bethesda, MD).(16) Arcs represent the total production of each cytokine, pie slices the polyfunctional capacity of cells. For the functional analysis of CD8+ T cells, that in theory can provide 64 populations of cells producing different combination of cytokines, a threshold of 0.5% was set on the basis of the distribution of negative values generated after background subtraction. Note that, as expected, in patient and control no CD8+ T cell was able to produce IL-2. [Color figure can be viewed at wileyonlinelibrary.com]

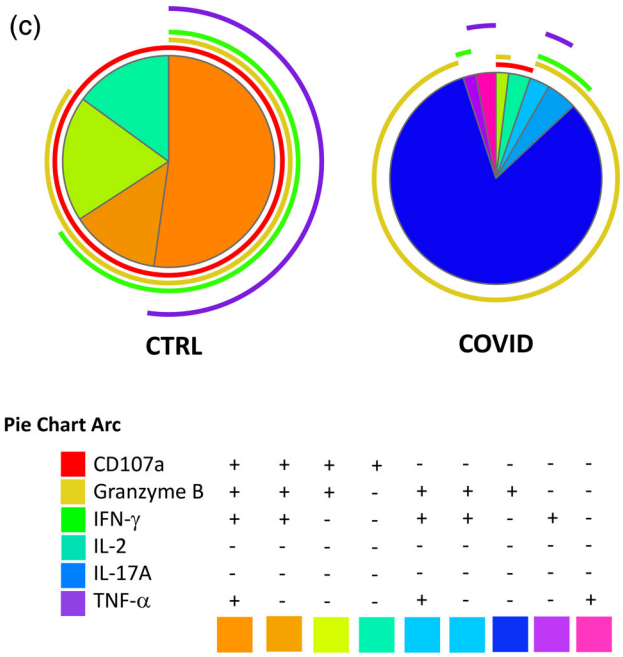

Figure 1. (Continued)

main personal protective equipment and collective protection devices are summarized.

Detecting Cells Responsible for the Cytokine Storm

Short term stimulation is now assuming a pivotal importance in the fight against Covid-19. Indeed, several reports have described abnormally increased levels of cytokines in plasma from patients infected by SARS-CoV-2 (12), that has been defined "cytokine storm," similarly to what has been described in bacterial sepsis (13). This condition is driven by, and causes inflammation, and molecules like interleukin (IL)-1, tumor necrosis factor (TNF)-α, and especially IL-6 are strongly produced by a variety of cells. Likely, since most infected people remain asymptomatic, this is not happening in all infected individuals. Interestingly, it is now known that children and pregnant women usually experience a mild form of Covid-2 if not a fully asymptomatic one. These categories of persons are characterized by an immune response skewed toward a TH2 profile (i.e., activities of the so-called T-helper Type 2 cells), with a preferential production of cytokines like IL-4 and IL-10. Typically, production of the aforementioned inflammatory cytokines is a feature of TH1 cells. Thus, it could be of interest to investigate whether profiling immune cells for their ability to produce TH1 or TH2 cytokines could be useful in the management of Covid-19 patients. This in vitro assay is typically based upon isolation of PBMCs, stimulation with different *stimuli* (i.e., anti-CD3/CD28, superantigens like *Staphylococcus aureus* enterotoxins, phorbol myristate acetate plus ionomycin, peptide pools) and quantification of intracellular cytokines.

In the last weeks this assay has been extensively used from our group to study CD4+ and CD8+ T cells. The experimental procedures that we follow require that PBMCs have to be maintained for a few hours (or, in some cases, for 2 days) in an incubator, at 37°C in a humidified atmosphere with 5% CO_2. For the analysis of polyfunctionality by the detection of intracellular production of cytokines, PBMCs are thus incubated with different *stimuli* inside capped tubes. In these conditions, aerosol particles or droplets can be generated inside the tube. However, tubes are loaded in a Class II BSC, transferred to the incubator, kept for some hours, unloaded and treated with Brefeldin A under the BSC as described before, and reincubated. At the end of the last incubation period tubes are unloaded in a BSC. Then, cells are fixed, permeabilized, stained with mAbs, and acquired. It is to note that the dedicated incubator is also located in the same BLS-2 laboratory. In the case reported by Figure 1 (from a study that has been approved by the "Area Vasta Emilia Nord" Ethical Committee on March 10, 2020), we used the Attune NxT acoustic flow cytometer (ThermoFisher Scientific, Eugene, OR). For this type of analysis, we first applied the classical methods for intracellular staining and rare event detection (14,17). Then, we found that a relevant difference in cytokine production was present between CD8+ T cells from a patient with Covid-19 pneumonia and an age- and sex-matched donor. Indeed, most CD8+ T cells from this patient were able to produce Granzyme B but not interferon-γ or TNF-α, and were CD107a negative. This type of assay is now under deep investigation to understand the clinical importance of a polyfunctional response that in viral infections like that by HIV plays a major defensive role and can predict, at least in part, the course of the infection (18,19).

Finally, we underline that sorting of cells from Covid-19 patients requires a completely different approach. In fact, the simple procedures that we have described above are easily applicable to studies where cells are finally fixed, like those on cell phenotype or detection of intracellular molecules, or other assays. For unfixed, living cells (as, e.g., in the case of analysis of the functionality of different organelles or of calcium fluxes, among others) we recommend to use the same measures required for cell sorting. At this regard, the ISAC Biosafety Committee has just released (March 26, 2020) novel procedures recently approved by the NIH-Institutional Biosafety Committee for CoV-2 cell sorting. The procedures are extremely clear and well written, and we invite those interested in visiting ISAC website at: https://isac-net.org/page/Biosafety.

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ISAC CYTOMETRY
Journal of Quantitative Cell Science PART A

Procedures for Flow Cytometry-Based Sorting of Unfixed Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Infected Cells and Other Infectious Agents

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Abstract

In response to the recent COVID-19 pandemic, many laboratories are involved in research supporting SARS-CoV-2 vaccine development and clinical trials. Flow cytometry laboratories will be responsible for a large part of this effort by sorting unfixed antigen-specific lymphocytes. Therefore, it is critical and timely that we have an understanding of risk assessment and established procedures of infectious cell sorting. Here we present procedures covering the biosafety aspects of sorting unfixed SARS-CoV-2-infected cells and other infectious agents of similar risk level. These procedures follow the ISAC Biosafety Committee guidelines and were recently approved by the National Institutes of Health Institutional Biosafety Committee for sorting SARS-CoV-2-infected cells. © 2020 International Society for Advancement of Cytometry

Key terms

biosafety and cell sorting; infectious cell sorting; ISAC Biosafety Committee; SARS-CoV-2 cell sorting procedure; COVID-19; coronavirus

Flow cytometry is a critical tool in clinical laboratories for diagnosis and monitoring of patients under various disease states, identification and characterization of infectious disease agents, and vaccine development (1,2). Recently, Cossarizza et al. (1) discussed specific approaches using flow cytometry for the study of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the novel human coronavirus responsible for the current coronavirus disease 2019 (COVID-19) pandemic. Flow cytometry facilities and laboratories, however, need to follow specific practices and procedures to prevent exposure of laboratory personnel to infectious agents. In a second recent paper, Cossarizza et al. (3) describe protocols and procedures for the safe handling, preparation, and flow cytometry analysis of fixed samples derived from blood (e.g. peripheral blood mononuclear cells) collected from COVID-19 patients. Here we describe an infectious cell sorting procedure for use with unfixed samples that follows the International Society for Advancement of Cytometry (ISAC) Biosafety Committee Guidelines and was recently approved by the National Institutes of Health (NIH) Institutional Biosafety Committee (IBC) specifically for sorting SARS-CoV-2-infected cells by flow cytometry. In this specific case, convalescent blood samples from COVID-19 patients, who were determined to be polymerase chain reaction (PCR) negative, was approved for these procedures. This protocol is also appropriate for blood samples containing other respiratory disease-causing agents such as pulmonary *Mycobacterium tuberculosis*.

Sorting flow cytometers have been shown to produce high concentrations of aerosols during partial nozzle obstructions or other malfunctions that disrupt the defined droplet pattern and stream trajectory and cause the stream to impact a hard surface (4,5). The size and concentration of aerosol particles depend on the sheath pressure, with a greater potential for release of high concentrations (up to 25,000 particles/cm^3) of small (1–3 μm) aerosol particles at high (≥70 psi) pressures (4). Aerosol particles in this size range are problematic because they are more likely to deposit in lung alveoli, are associated with increased infectivity of some organisms, and can remain airborne almost indefinitely (6–11). Sorting flow cytometers generally pose more of a risk to operators as the stream is not fully enclosed, and the instrument must be opened to retrieve the sorted samples. Although no known laboratory-acquired infections (LAIs) have been linked to flow cytometry, the cause of many LAIs is unknown and presumed to be transmitted through aerosols (5). A recent study of SARS-CoV-2 suggests that measures to contain viral spread should focus on droplet (i.e., airborne) rather than fomite-based transmission (12). Another recent report suggests treating this virus as airborne, even though current evidence remains inconclusive and the infectious dose is unknown (13). Thus reducing or eliminating aerosolization of SARS-CoV-2 samples and providing sufficient containment for procedures at risk of generating aerosols is critical for preventing LAIs of this and other similar agents.

To reduce the biohazard exposure of instrument operators using high-speed sorting flow cytometers, the ISAC Biosafety Committee created safety guidelines for the sorting of unfixed samples (5,14,15). Recommendations for managing aerosol generation by sorting flow cytometers include operating instruments at lower sheath pressures (e.g., <70 psi); directly evacuating the sort chamber through a high-efficiency particulate air (HEPA) or ultralow particulate air (ULPA) filter using an aerosol management system (AMS), aerosol management option (AMO), or other aerosol evacuation system; enclosing the flow cytometer within a primary containment device such as a Class II biosafety cabinet (BSC); and locating the flow cytometer in a dedicated room with restricted access and negative airflow. Specific engineering controls and procedures required for operator safety for particular institutions, workflows, or procedures should be determined using a risk assessment in collaboration with the IBC, Health and Safety Department, or other local biosafety office.

Several organizations, including the World Health Organization (WHO), the US Centers for Disease Control and Prevention (CDC), and the American Biological Safety Association (ABSA), have recently released general laboratory guidelines for working with SARS-CoV-2 samples which include some references to flow cytometry and cell sorting (16–18). These guidelines include:

1. Conduct an *institutional or local risk assessment* to ensure all procedures and analyses can be performed safely with appropriate risk control measures in place.
2. Follow good microbiological practices and procedures.
3. Perform *nonpropagative laboratory work*, including flow cytometry-based cell sorting of fixed or inactivated samples, at the biosafety level (BSL)-2 level. Procedures with a high likelihood of *generating droplets or aerosols* should be performed within a BSC or other primary containment device or should include additional barrier precautions for personnel such as surgical masks or face shields. Restrict access to the laboratory when work is being conducted.
4. Based on the risk assessment, perform flow cytometry-based cell sorting of unfixed samples at the BSL-2 level with enhanced precautions (also called BSL-2/3 or BSL-2 with BSL-3). All samples should be opened inside a BSC or other primary containment device. Additional personal protective equipment (PPE) should be used including respiratory protection (e.g. N95, N100, or powered air purifying respirator [PAPR]), double gloves, and eye protection.
5. Based on the risk assessment, perform high speed cell sorting, *propagative work*, and work with high concentrations of live virus at the BSL-3 level with inward airflow and a HEPA-filtered facility exhaust system. All sample manipulation should be done within a BSC or other primary containment device, and respiratory protection (N95 or greater) and face/eye protection is required.
6. Surface disinfect using appropriate disinfectants and contact times at every step. Appropriate methods and practices for management of all laboratory waste should be available in the facility.
7. Require *training of laboratory personnel* in handling pathogenic agents and for each specific procedure to be performed.

The above guidelines from WHO, CDC, and ABSA provide a general guide for handling SARS-CoV-2 samples in the laboratory. Here we expand on these guidelines and provide detailed procedures for all biosafety aspects of sorting unfixed SARS-CoV-2-infected cells and other respiratory disease agents. These procedures cover microbiological practices (laboratory setup, sample handling, decontamination, training of personnel), special practices for sorting flow cytometry (aerosol management and PPE), and infectious cell sorting. It is important to note that the protections and guidelines required to properly and safely carry out these procedures highlight the importance of the risk assessment and the involvement of each local biosafety office and/or IBC. At the NIH Vaccine Research Center (VRC), COVID-19 patient samples (PCR negative) can safely be sorted in a BSL-2 laboratory with certain BSL-3 practices in place during the sort (sometimes referred to as BSL-2/3). Three factors are critical among these practices: (1) the use of an AMS/AMO, (2) respiratory protection for the operator at times when the cell sorting chamber must be opened, and (3) a standard operating procedure (SOP) detailing the procedure in the event of a nozzle obstruction. See Holmes et al. (5) for guidance and details on performing a risk assessment, assigning a BSL, developing a SOP, and general recommendations for sample handling and processing for flow cytometry at each BSL.

Microbiological Practices for Sorting Flow Cytometry

General good microbiological practices required for the NIH VRC flow cytometry laboratory are outlined below. We recommend all work with SARS-CoV-2 and similar respiratory disease agents (BSL-2/3 and BSL-3) be performed in laboratories with negative airflow and filtered exhaust. The US Environmental Protection Agency List N includes all disinfectants approved for use against SARS-CoV-2 (https://www.epa.gov/pesticide-registration/list-n-disinfectants-use-against-sars-cov-2). Disinfectants that are on EPA lists traditionally achieve a minimum of a 3 log reduction of the targeted pathogen. For additional guidance on disinfectants, contact time, sharps, and hazardous waste management see Holmes et al. (5), Kampf et al. (19), and Biosafety in Microbiological and Biomedical Laboratories (BMBL) (20) and work with your local IBC or Health and Safety Department.

1. Good microbiological practices and procedures (16,20) should be followed including: never mouth pipette; never eat, drink, apply cosmetics, or handle contact lenses in the laboratory; appropriately handle and dispose of sharps; protect street clothing by wearing appropriate PPE or using dedicated laboratory clothes and shoes; perform all procedures in such a way as to minimize the creation of aerosols; clean work surfaces with an appropriate disinfectant after working with infectious materials; decontaminate or sterilize infectious laboratory waste before disposal.
2. Verify all laboratory rooms are under negative airflow using an appropriate air flow indicator such as Ball-in-the-wall® (Airflow Direction Inc., Newbury, MA; Supporting Information Fig. S1).
3. Gloves should be worn at all times within the flow cytometry facility. When leaving a contaminated area, gloves can be wiped with a disinfectant (at least 30 s of contact time) or removed and replaced with new gloves.
4. Conduct all open manipulations involving infectious materials in BSCs or other primary containment devices.
5. Clean and decontaminate BSCs and other contaminated equipment and items used in the protocol with an appropriate disinfectant after each use, including removal and proper disposal of consumables and biohazardous waste.
6. Decontaminate all work surfaces after use, at least once per day, and after any spill of contaminated material. Work surfaces can also be decontaminated prior to starting work.
7. Hypochlorite of 10% (bleach) can be used as a primary disinfectant. Note that 10% bleach (10 ml hypochlorite in 90 ml water) stored in an opaque plastic container at room temperature will lose effectiveness upon exposure to light and air. It is recommended that 10% bleach solution be made fresh daily. Alternatively, hydrogen peroxide can be used as a primary disinfectant. Peroxigard (Virox Technologies, Inc., Oakville, ON, Canada), which is an accelerated hydrogen peroxide formula, was found to kill SARS-CoV-2 within minutes after contact (21,22). 10% betadyne (10 ml concentrated betadyne in 90 ml water) and 70% ethanol (70 ml ethanol in 30 ml water) can be used as secondary disinfectants.
8. It is recommended that access to the flow cytometry laboratory is restricted when infectious materials are in use. Appropriate signage should be posted when access is restricted indicating any special precautions required before entering the room. Signage can also include the agent(s) in use and the name(s) and contact information of responsible individuals. See Supporting Information Figure S2 for example signage indicating whether a sort is in progress or "active."
9. Biological spills can be decontaminated with 10% hypochlorite (bleach) or other appropriate disinfectant. If a spill occurs within a primary containment device, apply absorbent toweling to the area, and soak with an appropriate disinfectant. Allow 30 min of contact time before cleaning with additional disinfectant applications. Dispose of all contaminated materials as biohazardous waste. Spills of biohazardous materials outside of primary containment generally necessitate evacuation of the work area and/or laboratory until aerosols are cleared from the room.
10. Biological spills and accidents that result in overt or potential exposure to infectious materials must be reported. Appropriate medical evaluation, surveillance, and treatment must be provided and written records maintained.
11. Proper procedures must be followed for transport of infectious materials within and outside of the laboratory. These procedures generally include placing sealed samples within a labeled secondary leak-proof container, and disinfecting the outer container. Samples that have been inactivated using an approved method (e.g., fixation with formaldehyde solution) can be handled as non-infectious. Note that 0.7% and 1% formaldehyde were shown to effectively inactivate SARS CoV and render it non-infectious after 30 s of exposure (23).
12. Contaminated equipment must be decontaminated using a primary and secondary disinfectant before removal from the facility for repair or maintenance or packaging for transport, in accordance with the applicable local, state, or federal regulations. Service engineers entering the facility must be instructed on the appropriate protective clothing required by the facility.
13. All personnel should receive appropriate training on the potential hazards associated with various procedures, the necessary precautions to prevent exposures, and the exposure evaluation procedures. This training should be updated annually or on a regular basis, and additional training should be provided as necessary for procedural changes.
14. It is recommended that laboratory personnel receive appropriate immunizations or tests for the agents handled or potentially present in the laboratory, if available.

Table 1. PPE and additional protections and procedures required at each biosafety level at the NIH VRC; see infectious cell sorting procedure for more details about BSL-2/3

	BIOSAFETY LEVEL BASED ON RISK ASSESSMENT		
TYPE OF PROTECTION	BSL-2	BSL-2 PLUS	BSL-2/3
Aerosol containment testing	Monthly	Weekly	Each Sort
Tyvek suit (full body)	Not required	Optional	Required
Lab gown (closed front)	Required	Required	(Tyvek only)
Latex gloves	Single	Single	Double
Respiratory protection (e.g., PAPR)	Optional	Required	Required

PPE and additional procedures recommended for flow cytometry laboratories at the NIH VRC are based on the BSL as determined through a risk assessment (Table 1).

Special Practices for Sorting Flow Cytometry

Aerosol Management System

The AMS/AMO consists of a hose or series of hoses attached to openings in the sort chamber or sort collection area that are connected to the blowers of a BSC (for integrated systems) or to an external-filtered vacuum source such as a Buffalo Filter® (ConMed, Utica, NY). Negative airflow is created inside the sort chamber, and the air is evacuated through the hose(s) into HEPA or ULPA filters within the BSC or the external vacuum source. While sorting viable infectious material (infected cells) under high pressure, the following guidelines must be followed for proper aerosol containment. All operators should be trained and certified by the flow cytometry facility prior to performing any cell sorting procedures.

1. The BSC (for integrated AMS/AMO) or the HEPA filter within the AMS/AMO (for external units) should be certified after installation and at least annually thereafter. Factory HEPA filter testing may reflect the integrity of the filter paper before the housing was constructed and may not account for damage during packaging and shipment.
2. The AMS/AMO or other aerosol evacuation system must be on and functioning according to the manufacturer guidelines. Supporting Information Figure S3 shows the aerosol flow and the locations of the vacuum gauge and monitor. For the BD FACSAria flow cytometer (BD Biosciences, San Jose, CA), the vacuum monitor should be set to 20%, and the vacuum gauge must read between 1.0 and 2.5 in. of water. The HEPA filter unit and tubing must be replaced after 6 months or if increased percentage is needed to achieve the required vacuum pressure.
3. The waste tank must contain enough hypochlorite to provide a final concentration of 10% when filled (e.g., 1 l household beach to a final 10 l of waste collected). If full, empty the waste tank before starting cell-sorting procedures. Allow at least 30 min of contact time before disposal.
4. The sort chamber camera system must be functioning according to the manufacturers guidelines. This camera system is used to monitor the sort stream and alerts the operator to potential increased aerosols. In this situation, the operator can correct the sort stream and reduce aerosol contamination. Some instruments, including the BD FACSAria, are equipped with a "Sweet spot" monitor, which should be used during all sorting operations. This device monitors drifts in the sort stream and corrects its position by automatically adjusting the wave amplitude. If a stream blockage is detected, the Sweet spot monitor will automatically shut down the stream and close the sort drawer.

Measurement and Tolerances of Aerosol Containment

The aerosol evacuation system must function properly to contain aerosols released during a partial nozzle obstruction or other instrument failure. Aerosols generated during a simulated partial nozzle obstruction were successfully contained when a BSC with an AMS was used, and the AMS was considered a critical component of these engineering controls (4,24). The ISAC guidelines recommend verifying containment of aerosols by the AMS/AMO on a regular basis (as determined by a risk assessment and assignment of BSL) and before working with potentially infections or hazardous samples (5). Proper function of the AMS/AMO is generally not verified during annual BSC certification, and instruments that were not installed within a BSC can use an external aerosol evacuation system. Therefore, aerosol containment must be verified using an independent assay. Perfetto et al. (25) describes the method currently recommended by the ISAC Biosafety Committee to verify containment of aerosols by sorting flow cytometers. In this method, a "worst case scenario" is simulated by creating a large aerosol release inside the sort chamber, the aerosol particles are labeled with small (1 μm) fluorescent beads, and air samples are collected using a disposable air sampler designed to efficiently collect particles in the 1–3 μm size range in an attempt to detect the labeled aerosol particles. Aerosol containment is typically verified bi-monthly unless otherwise indicated by the risk assessment evaluation. Containment must also be verified after initial installation, removal of the instrument from the BSC, and any maintenance or repairs performed on the AMS/AMO or any of its components. Steps to verify aerosol containment of the BD FACS Aria are outlined below.

1. Aerosol containment testing should be performed at 70 psi or at the maximum sheath pressure used for all workflows. Analysis and sorting utilizing lower sheath pressures than that used during the containment test can be performed without additional containment testing. However, if higher sheath pressures will be used, the aerosol containment test must be repeated to verify that the additional aerosols released at these sheath pressures are contained.
2. Turn on the AMS (20%), and check for proper vacuum function (1.0–2.5 in. of water).
3. Prepare a sample of 1 μm Dragon Green beads (Bangs Laboratories, Fishers, IN; Supporting Information Fig. S4) such that an event rate of 40,000–50,000 events/s can be achieved. Set the trigger detector to green fluorescence (i.e., fluorescein isothiocyanate (FITC) channel). For the BD FACSAria set to 70 psi sheath pressure, add 20 μl of concentrated bead solution to 2 ml of buffer (phosphate buffered saline with 0.2% sodium azide and 0.5% Tween 20). Run the prepared bead sample at a flow rate of 5 or 6 to achieve the desired event rate. For other instruments and/or sheath pressures, adjust the sample flow rate and/or the bead concentration as needed to achieve the desired event rate. Supporting Information Figure S4 (top panels) shows an example of a scatter plot and histogram of the Dragon Green bead sample.
4. Create an aerosol release to simulate an instrument failure such as a partial nozzle obstruction. For the BD FACS Aria, this is accomplished by covering the waste catcher with a small piece of rubber tubing forcing the stream to glance off the waste catcher shield (Supporting Information Fig. S5).
5. Attach a cyclex-d cassette (Environmental Monitoring Systems, Charleston, SC) to a vacuum pump, and verify the vacuum pump is set to 20 l/min. Place the cassette toward the front of the sort chamber approximately 5 cm (2–3 in.) from the sort block door. For the BD FACSAria, close the sort block door but do not install tube holders (Supporting Information Fig. S6). The main sort chamber should also be closed.
6. Click on sort drawer to retract, which will begin creating aerosols as the stream hits the rubber tubing covering the waste catcher (Supporting Information Fig. S5). Note: It is recommended that the operator wear respiratory protection (e.g., N95, N100, or PAPR) while aerosols are being generated.
7. Turn on the vacuum pump and collect an air sample for 10 min.
8. Turn off the vacuum pump and attach a fresh cyclex-d cassette. Turn the pump back on, and collect a positive control sample by sampling for 2 min with the AMS turned off. Both the sort block door and the sort chamber door should remain closed.
9. After all air samples have been collected, turn the AMS on and return the waste catcher to its normal position. Remove the rubber shield from the waste catcher.
10. Remove the cover slips from the cyclex-d cassettes (Supporting Information Fig. S7). Place each cover slip on a gridded microscope slide adhesive side down ensuring the beads and grid lines are in the same focal plane. In an extreme example of aerosol escape, a faint circle of dried PBS can be seen in the center of the slide (Supporting Information Fig. S7).
11. Examine the entire adhesive region for the presence of Dragon Green beads using a fluorescent microscope with a FITC filter (520–640 nm, see Supporting Information Figs. S4 and S7). Scan the slides using a 10× or 20× objective, and count all beads present.
12. The acceptance tolerances are zero Dragon Green beads detected after 10 min of active air sampling in front of the sort chamber door with no tube holder in place and the AMS turned on. The positive control slide must contain greater than 100 beads after 2 min of active air sampling with the AMS turned off and no tube holder in place.
    i. If beads are observed on the test slide, aerosol containment has NOT been verified. The operator should check all vacuum tubing and that the correct settings have been used on the instrument, and repeat the test.
    ii. If the test fails twice, infectious cell sorting must be aborted until aerosol containment can be verified. Contact the manufacturer, if needed, to perform corrective maintenance or repairs.
13. See Supporting Information Appendix S1 for an example checklist form for aerosol containment testing.

Infectious Cell Sorting Procedure

All laboratory practices using nonamplified specimens containing SARS-CoV-2, *M. tuberculosis*, and other agents within this risk group must be performed using the following guidelines in accordance with the CDC recommendations as outlined in BMBL (20). See Supporting Information Appendix S2 (BD FACSAria II), Supporting Information Appendix S3 (BD Influx, BD Biosciences, San Jose, CA), and/or the manufacturer's instructions to properly start-up the flow cytometer.

1. Procedures involving samples containing live respiratory disease agents must be done at BSL-2/3 or higher.
    i. Respiratory protection (e.g., N95, N100, or PAPR) must be worn for all procedures involving live respiratory disease agents. See Supporting Information Appendix S4 for a description of the AirMAX HEPA filter PAPR (Bio-Medical Devices Intl, Inc., Irvine, CA) and an example checklist for starting work in the BSL-2/3 laboratory including inspection of the PAPR and laminar hood, verification of room pressure, and verification of an aerosol containment test.
    ii. Other recommended PPE includes double gloves, Tyvek suit (see image in Supporting Information Appendix S4) and/or dedicated laboratory clothing, shoe covers and/or dedicated laboratory shoes (not required if wearing Tyvek suit with integrated shoe covers), solid front disposable lab coat (not required if wearing Tyvek suit), disposable sleeves,

and eye protection such as safety glasses or face shields (not required if wearing a PAPR).
iii. Infectious disease agents must be handled inside a certified BSC or other primary containment device.
iv. It is recommended that the entire flow cytometer be placed in a BSC or other primary containment device with an integrated aerosol evacuation system.
v. Aerosol containment must be verified before every sort.
vi. Procedures with the potential to generate aerosols should be performed inside a BSC. See Supporting Information Appendix S5 for centrifugation procedures.
vii. When exiting the laboratory, all disposable PPE should be disposed as hazardous waste. All reusable PPE (e.g., safety glasses, PAPR hood) should be disinfected with 10% bleach or other approved disinfectant.

2. The flow cytometer must pass all tolerances of aerosol containment as described above. If these tolerances are not met, infectious cell sorting is not permitted.
3. The operator must wear PPE as outlined above. If the operator is not protected as described in this section, infectious cell sorting is not permitted.
4. A warning sign must be posted outside of the flow cytometer laboratory (see Supporting Information Fig. S2), and the room is limited to two individuals during the sort procedure.
5. Turn on and verify that the AMS is working correctly. For the BD FACS Aria, this device must have a vacuum pressure of 1.0–2.5 in. of water. If this tolerance is not met, infectious cell sorting is not permitted.
6. Close all barriers around the sort chamber. If this is not done, infectious cell sorting is not permitted.
7. All samples must be filtered through an appropriate sized filter (depending on the cell size and nozzle size) prior to sorting, and filtering must be done inside a BSC or primary containment device. For lymphocytes, a 40-μm mesh is generally recommended. This reduces the potential for clogging and decreases the risk of creating aerosols.
8. Monitor the sort performance using an internal camera such as the Accudrop camera. If during the sort the stream is deflected (due in part to a clogged flow cell tip), the BD FACS Aria is designed to stop automatically and block the sort tubes. The sort will not restart until the operator has cleared the clog. Use the following procedure to remove a clog from the cytometer.
   i. Remove the sample from the sample chamber.
   ii. Turn the stream off (unless turned off by the instrument in the automated shut-down mode) and then on again to see if drop delay and stream returns to normal pattern.
   iii. If the obstruction is not removed by turning the stream off and on, wait 2 min and remove the sample collection tubes. Close the sample chamber, retract the sort drawer, and wait for an additional 2 min. This will allow any aerosols inside the sort block to be evacuated before the sort block door is opened. Remove the clogged nozzle and either replace with a new nozzle or decontaminate the nozzle in 10% bleach for 30 min before placing in a sonicator (2–5 min).
   vi. Before reinserting the nozzle, check for a clear nozzle hole using a microscope. Dry the nozzle slot, stopcock, and deflection plates using a Kimwipe or other lint-free toweling if necessary.
   v. Sorting can be resumed after the clog is cleared from the original nozzle or it is replaced with a new nozzle. Repeat the procedure to verify droplet location and proper drop delay.
9. Do not remove any samples from the sort chamber until sample acquisition has been stopped, and wait 2 min before opening the sort chamber door. This procedure will clear aerosols from the sort chamber. After this time, sorted samples can safely be removed from the sort chamber.
10. When the sort is finished, proceed with the flow cell disinfection procedure and shutdown as listed in Supporting Information Appendix S2, Supporting Information Appendix S3, or following the manufacturer's guidelines. Follow site-specific biosafety procedures for proper doffing of PPE.

Summary

Laboratories involved with vaccine development will be required to sort unfixed SARS-CoV-2-infected cells. Although sorting flow cytometers produce little to no aerosols under normal operation, the potential exists for release of high concentrations of aerosols if instrument failures occur (4). Containment of these aerosols is essential for operator safety when working with potentially infectious or otherwise hazardous samples, especially with sorting flow cytometers where the fluidics path is not entirely enclosed and which must be opened to retrieve sorted samples. Aerosol containment is accomplished through the use of primary containment devices, such as BSCs, and direct evacuation of the sort chamber or sort collection area using an AMS/AMO. Finally, when working with samples known to contain an infectious agent at a high concentration (e.g. bronchial lavages), a risk assessment must be performed to determine if a procedure should be done within a BSL-3 laboratory, or at a minimum with the sorter operational and certified within its own BSC or other primary containment device within a BSL-2 laboratory. This decision to increase the safety parameters (and likely the cost) is made to further lower the risk of potential exposure to personnel during the sort. Recommendations under the BSL-2/3 level as defined in the recent SARS-CoV-2 procedures are summarized below:

1. Review a risk assessment plan with your biosafety representative, such as the Institutional Biosafety Officer and/or the IBC.

2. Test aerosol containment prior to cell sorting using the cyclex-d procedure to validate instrument containment of aerosols while sorting.
3. Perform cell sorting with an instrument equipped with an operational and HEPA-filtered AMS/AMO.
4. Required use of PPE: Tyvek full body suit, gloves and shroud with HEPA-filtered PAPR to be used at particular times during the sorting procedure.
5. Measurement check for negative room air flow.
6. Clean surfaces before and after sort with 70% ethanol, 10% hypochlorite, or other approved disinfectant.
7. Maintain records of containment measurement and a safety checklist.

Implementation of these biosafety practices during the handling and sorting of risk agents such as SARS-CoV-2 will ensure that laboratories maintain a high level of public safety during vaccine development and deployment.

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