Cyt-Geist: Current and Future Challenges in Cytometry: Reports of the CYTO 2018 Conference Workshops

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CONTENTS

ORGANIZED by the International Society for Advancement of Cytometry (ISAC), the CYTO conference is one of the most important events for everyone interested in cytometry and quantitative single cell analysis. It is an annual assembly of cytometry experts as well as novices in this field—all having one common denominator—passion for cytometry. This meeting is not only unique possibility to learn new, exciting cutting-edge research directly from the source by attending the Frontiers and the State-Of-The-Art lectures, research, and technology sessions, or Tutorials.

CYTO conferences are also the best possible live forum to discuss current and emerging challenges in cytometry associated sciences at the utmost interactive CYTO formats—the Workshops. The Workshops attract a lot of attention within and outside the cytometry community as they generally discuss where we stand in specific fields of interest, how problems can be addressed and solved, and what are the future development areas and demands. Due to limited time frame allocated to this format; however, the workshops are run in parallel so sometimes attendees cannot participate in all the workshops of their interest. Moreover, those who could have not join the CYTO conference miss the content entirely. Therefore, we decided to summarize all the workshops to disseminate the information provided and the state-of-the art view of the community. Thus, the main reason for creating such combined summary of reports was to keep the record of the live discussions and main conclusions as well as to propose eventual guidelines and make them available to the broader public.

This manuscript serves as a summary report of 16 workshops (WS) that were held at CYTO2018 in Prague. We present in concise form the current and the future challenges in cytometry identified by workshop organizers. The manuscript is divided into four topic chapters: Trends, Shared Resource Laboratory (SRL) Best Practices, Quality Assurance and Reproducibility, and Technology. Each chapter is a summary of four workshops. We intend to serve with this joint workshop report the global community involved in single cell analysis and cytometry.

CHAPTER 1: TRENDS

Four workshops that focused on current trends in cytometry are gathered in this chapter. While each workshop had a different format and different goals, together they cover a wide range of interests across the community in identifying future trends in cytometry. Several trends in flow cytometry were reviewed regarding increasing activity and unmet challenges in the field of nanoparticles measurement, chip-based cell sorting, spectral flow cytometry, evolution of mass cytometry, and challenges in high-dimensional data analysis (WS18).

The relationship between flow cytometry and genomics discussion exploring ways to engage the cytometry community with initiatives aimed at single-cell genomic analysis, such as the comprehensive Human Cell Atlas sequencing program, seeking to derive mutual benefit from the exchange was

started (WS06). The initiative to embrace and leverage the field of open innovation in cytometry, a new ISAC Open Science group, CYTO Lab Hacks, was launched, with the goal to serve as an open, transparent, and accessible forum for innovation exchange in cytometry (WS07). The use of flow cytometry to generate identifying "fingerprints" of microbial communities was discussed. The awareness of microbial community flow cytometry was raised, and links with practitioners in other fields were established (WS15).

WS18: FLOW CYTOMETRY TRENDS AND DRIVERS

Giacomo Vacca, Nao Nitta.

Introduction and Aims

The intended audience was research managers, instrumentation developers, flow cytometry core laboratory directors, principal investigators, technologists, engineers, investors, market analysts, and engineering students. The main goal of the workshop was to give attendees a look at some of the most advanced solutions and challenging applications in flow cytometry (FCM). A second goal was to engage participants in a discussion aimed at gaining a broad consensus on the areas of greatest innovation and of greatest unmet need in FCM. Five FCM experts were chosen to present retrospectives on key innovations or subjects that have had, or are expected to have, a significant impact in the field. 1. Dr. John Nolan, of the Scintillon Institute, talked about the challenges of measuring Extracellular Vesicles (EVs). 2. Dr. Martin Büscher, of Miltenyi Biotec, discussed Micro-Electro-Mechanical System (MEMS) sorting. 3. Dr. Michael Zordan, of Sony Biotechnology, addressed spectral FC. 4. Dr. Dmitry Bandura, of Fluidigm, talked about mass cytometry. 5. Dr. John Quinn, of FlowJo/BD Bioscience, discussed high-dimensional data analysis.

Methods

The facilitators started off the workshop with a discussion of the framework—the interplay of trend drivers (applications) and trend enablers (technology). The following examples of each were provided:

- Drivers
  - smaller systems
  - smaller particles
  - greater multiplexing
  - richer information
- Enablers
  - microfluidics
  - on-chip sorting
  - acoustic cytometry
  - higher power lasers
  - dense wavelength coverage
  - higher sensitivity
  - spectral resolution
  - tunability
  - novel probes
  - waveform analysis

- fluorescence lifetime
- light scattering

Each of the speakers was given 12 min to present on their assigned topic; each presentation was followed by Q&A with the audience. After the last presentation, the audience was invited to ask additional questions and provide feedback to the facilitators. The five presentations (available as Supplementary Information WS18_SI1–SI5), as well as questions from the audience and answers from the speakers, were collated and summarized.

Results/Outcome

1. The presentation on EVs by Dr. John Nolan (see Supplementary Information WS18_SI1) highlighted current challenges of measuring very small and dim particles. The size distribution of EVs is below what most flow cytometers can detect, either by scattering or fluorescence. Unmet needs include more sensitive instrumentation, brighter and smaller antibody labels, and new standards and controls.

2. Dr. Martin Büscher's talk on MEMS sorting (see Supplementary Information WS18_SI2) discussed the fundamentally different sorting principles in traditional droplet sorting, microfluidics "pulse" sorting, and microfluidics MEMS sorting. Since MEMS sorting is nonresonant, it can precisely adjust the sorting window in each event to improve the purity and yield, which is impossible with conventional resonant-based droplet sorting.

3. The discussion by Dr. Michael Zordan (see Supplementary Information WS18_SI3) reviewed the key elements of spectral FC: continuous dispersion of the collected fluorescence; multichannel array detectors; and unmixing of the resulting spectral signatures. By collecting full spectra, a spectral flow cytometer can better distinguish fluorophores with overlapping emissions: examples were presented: QD605 vs. BV605 and GFP vs. AF488. Other discussed benefits included extraction of autofluorescence signals and simplification of panel design.

4. Dr. Dmitry Bandura's presentation on mass cytometry (see Supplementary Information WS18_SI4) showed the growth in the number of "colors" available using metal tags (37 at latest count) versus fluorescence (28). With the increasing number of parameters, the need arises for more sophisticated and intuitive data analysis tools, such as Cytobank (Cytobank, Santa Clara, CA) and GemStone (Verity Software House, Topsham, Maine). Cell detection efficiency, which initially (at 6%) was far below the capabilities of routine FC (essentially 100%), has increased to 50%. An extension of mass cytometry that analyzes and images cells on slide smears were also presented.

5. High-dimensional data analysis was discussed by Dr. John Quinn (see Supplementary Information WS18_SI5), with an emphasis on unmet needs in protein analysis. FC is not yet able to provide anything close to a full characterization of single-cell proteomes (20,000 proteins). Another important aspect of cell systems underserved by current tools is analyte heterogeneity. Using DNA barcodes instead of fluorescence tags as antibody conjugates can increase the number of measurable parameters. This in turn creates a need for better analysis tools such as automated data cleaning, dimensionality reduction, clustering, and deep learning.

Discussion/Conclusion/Perspectives

The workshop reviewed technical advancements and expansion of FCM applications. A central theme that emerged during the workshop, both in the presentations and in the discussions, is the importance of the technology–application coevolution. There was general agreement among the participants that in this area the specific demands from science and industry, or market drivers, foster engineering efforts to develop new instruments, reagents, and software. For example, the strong demand from biology to measure EVs is challenging the cytometry community, while emerging technologies such as mass cytometry and spectral FCM are developed to fulfill the demand to increase the number of detectable parameters.

Abridged questions from the audience, and the speakers' answers, included:

- EVs
  - Q: Any analysis challenges specific to EVs?
  - A: Same as for cells, except for the greater Poisson noise inherent in signals from small particles.
  - Q: Would nonoptical technologies be superior to optics given optical limitations in measuring very small particles?
  - A: Optics is the only technology we have that, beside size, can measure cargo.
  - Q: Besides sensitivity, what are some of the critical considerations in EV measurements?
  - A: Coincidences ("swarming"); contamination; sorting effectiveness; better standards (microspheres are not ideal).
- MEMS Sorting
  - Q: What are some of the advantages of MEMS sorting?
  - A: Greater flow stability of the self-contained closed system, enabling long sorts (hours). Sheathless sorting allows multistage sorting, compounding the enrichment factor.
  - Q: What are the boundaries of the performance triangle (purity/speed/yield) achievable with MEMS sorting?
  - A: Depends on the nature of the sample. For a typical sample, at about 95–96% purity, speed is 5–10 k events/s.
- Spectral Flow Cytometry
  - Q: Smaller spectral slices mean poorer statistics. Given the tradeoff between sensitivity and the spectral width of channels, what have you found to be the optimum number of channels in spectral FC?
  - A: 32. Sixteen channels do not give enough resolution, 64 give diminishing returns due to extra noise.
  - Q: What are the rules for designing a panel for a spectral FC?

- A: If the fluor spectra are more than 5–10 nm apart, you should be pretty safe; large differences in the widths of overlapping spectra (e.g., BV and quantum dots) also help with unmixing.
- Mass Cytometry
  - Q: What are some of the limitations of mass cytometry?
  - A: Given that isotopic abundance is not always 100%, sensitivity can suffer as a result. Isotopic spillover interference is at the 1% level. Oxides from poor plasma quality are also potential sources of error, as is contamination. Analysis rates are currently about 500 events/s (max 1,000 events/s). The fundamental physical limit (a couple of kHz) is due to the duration of the ion cloud in the plasma.
  - Q: What are the best current yield efficiencies in mass cytometry analysis?
  - A: About 50% (100% with imaging mass cytometry).
- High-Dimensional Data Analysis
  - Q: How much of a learning curve is there with the very sophisticated new tools?
  - A: tSNE is relatively straightforward, clustering is more complex; but experimental setup is the most critical step in guaranteeing accurate analysis results.
  - Q: Why is tSNE gaining such popularity and is it warranted?
  - A: The emergence of high-dimensional analysis was caused by the growth in the number of accessible parameters in FC. tSNE is popular because it is relatively simple to learn and to use.

Many of the workshop participants agreed on the following:

- Detection of EVs is still challenging and currently researchers are taking several different approaches to quantify the amount and content of EVs.
- MEMS sorting has unique benefits, and it provides an additional option for researchers.
- Spectral flow cytometry is gaining popularity as it highlights new applications such as autofluorescence extraction.
- Mass cytometry is widely used by researchers since it meets the requirement to increase the number of parameters.
- As multimodal single-cell data become available through advancements in cytometry and genomics, data analysis platforms are also evolving to provide easy-to-use solutions to accelerate biological discoveries from big data.

The workshop helped assess the impact of recent advancement in FCM, contributed to an understanding of the issues faced by cytometry researchers as they deal with biological findings and their industrial applications, led to a full understanding of the challenges of emerging solutions, and, ultimately, illustrated how technological advancement is essential to further understand the nature of cells and promote the medical and industrial applications of cytometry.

WS06: CYTOMETRY IN THE ERA OF THE HUMAN CELL ATLAS

Robert Salomon, Christopher Hall, Thomas M. Ashhurst.

Introduction

Since the late 1960s, FCM has given the biologist the ability to interrogate both normal and diseased biological conditions at the single-cell level in a statistically relevant way (1). For many years, flow cytometry advancements were largely restricted to incremental improvements in laser numbers, laser powers, and the number of simultaneous detectors available. In recent years, the advent of high-dimensional fluorescence cytometers, such as the Symphony A5 (Becton Dickinson™), and mass cytometry, such as the Helios (Fluidigm©), has allowed us to push the dimension barrier above 18 parameters. This, however, is insignificant in comparison to the new raft of single-cell transcriptomics tools such as the InDrop, DropSeq, and Chromium 10X systems, which can analyze thousands of dimensions per cell (2). These systems are at the heart of initiatives such as the Human Cell Atlas (HCA) (3) an international endeavor to map every cell in the human body, and the Allen Brain Atlas (4,5). While these systems have initially been adopted by the genomics Shared Resource Laboratories (SRLs), there is no doubt that these systems are, in fact, simply specialized forms of cell sorters and are part of the rapidly expanding field of genomic cytometry.

Method

With the stated goal of understanding what the modern cytometrist requires to fully engage with genomic cytometry, we solicited opinions and comments in the form of a pre-workshop survey and a question-and-answer session. The survey design was intended to inform the workshop regarding the intended audience, challenges faced by cytometrists with regard to "Omic" technologies, and how they would like these challenges tackled.

The survey questions, which can be found in Supplementary Information WS18_SI1, were distributed using the cytometry communities on Google+, the Purdue mailing list and Twitter. The survey is still open and can be completed here: https://www.surveymonkey.com/r/G2SRHMW. Questions were open ended, allowing participants to freely engage with the subject matter and to be able to choose more than one answer if relevant. After introductory talks on the HCA, single-cell Omic technologies, and the integration of these technologies into the SRL, questions and opinions were taken from the audience.

Results

There were 19 responses to the pre-workshop survey and over 10 contributions from the audience during the workshop. Detailed responses from the survey and workshop can be found in Supplementary Information WS06_SI1 and 2, respectively, and an interactive dashboard of the survey results can be found on Tableau Public at https://public.tableau.com/views/Cyto2018workshop6/SurveyResults.

The survey respondents covered a diverse range of research with 8 specializing in flow cytometry, 4 in imaging, and 16 using Omic technologies. The focus of this research was on immune cells (12 responses), tissue samples (10 responses),

and other cell types (11 responses); split between human (8 responses), mouse (8 responses), and other species (4 responses). The majority of respondents cite a lack of knowledge and/or procedural issues as their number one challenge when engaging with Omic technologies (13 responses). This is reflected in the answers to "What can ISAC do to help you get into this space?" where 9 requested better access to training or to standardized protocols and 5 wanted greater access to expert collaborators and regional meetings.

Audience participation, during the workshop, reflected the pre-workshop survey with commentary on the need for better access to educational resources (comments 1, 2, and 4) and the need for more collaboration between fields, with an emphasis on the cytometry communities single-cell preparation experience (comments 2, 5, 6, 8, and 9). A synopsis of the workshop discussions can be found in Table 1.

Discussion

The HCA is the next logical extension following on from the Human Genome Project. The creation of a human cell atlas is a significant scientific and technical challenge and its success would be a great achievement. Currently, cytometry is largely separate from the efforts of the HCA and we, as a community, need to actively participate in this important work, and in the broader field of single-cell genomics.

Table 1. A synopsis of each comment or question during the workshop. A detailed description of each can be found in Supplementary Information WS06_SI2

COMMENT	SYNOPSIS
1	Need for consensus on methodology including the production of best practice documents.
2	Need for education. Cytometrists need to actively engage with HCA and not wait to be asked.
3	HCA is an iterative project and will need to analyze both resting state and stimulated cells.
4	ISAC should strengthen their educational material to include Genomic Cytometry. There needs to be a "multi-Omic" approach to future research.
5	ISAC should collaborate with other related societies.
6	Sequencing cores are not necessarily experienced in cell preparation. That is where cytometry cores will help.
7	Different types of SRL, what equipment should go where, and how they should interact.
8	There will still be a need for cell sorting to isolate rarer cells to analyze.
9	Genomic Cytometry will not steamroller traditional cytometry. Sorting and validation will always be required.
10	The current trend of scRNAseq followed by biological validation may not be the best method.

"Genomic cytometry" is the newest field of cytometry and involves using genomic sequencing techniques in order to measure the unique characteristic of individual cells. Using current techniques, it is possible to measure a multitude of cellular features including DNA content (scDNASeq) (6), RNA profiles (scRNASeq; Chromium10x) (7), Dropseq (8), seqwell (9), InDrop (10), Rhapsody (11,12), chromatin accessibility (ATACseq) (13), DNA methylation (CLEVERseq) (14), and protein expression (CITEseq) (15). Importantly, as all of these methods aim at providing measurements of the single cell using genomic technologies, they should be considered genomic cytometry methods.

There are many challenges in the use of genomic cytometry techniques. Some relate to the technology itself and others are more biological in nature. This is perhaps unsurprising, as the technology is still in its infancy. The challenges predominantly involve cell preparation, throughput, scalability, cost, the inability to interrogate the sample in real time, the size and complexity of the data being generated, and the destructive nature of the interrogation method. Other challenges that are more biological in nature relate to the instability of RNA, the discordance of RNA with protein expression, and lack of form or functional assessment when using scRNASeq alone (16,17).

The world of cytometry has a great deal to offer the HCA and related genomic projects. Figure 1 outlines how the world of traditional cytometry (fluorescence, mass, and imaging cytometry) interacts with the emerging field of genomic cytometry. These include, but are not limited to, expertise in cell preparation, cell sorting to isolate cells of interest, index sorting to match protein expression data to resulting sequencing data, and antibody staining methods. Additionally, work done in scRNAseq needs to be substantiated by investigating the corresponding measures of protein, form, and function (18).

Because the field is currently undergoing refinement, it is a dynamic and confusing space for the uninitiated as well as those that have been involved from early on. For example, the first challenge that many cytometry SRLs will face is where these new technologies should reside: Should they be placed in the genomics core, or in the cytometry core? There has been much discussion in the cytometry community regarding where the boundaries of these technologies lie. Many major institutions are also beginning to make these assessments and societies like ISAC are ideally situated to create a framework for these decisions and the emerging field of genomic cytometry.

As a cytometry community, we need to respond to the challenges inherent to the era of the HCA and become leaders in it. We need to move away from the concept of cytometry as predominantly a search for epitope-antibody interactions and to embrace cytometry's core purpose, the measurement of cells at the single-cell level. Once we embrace this concept fully, we can begin to embrace the new field of genomic cytometry.

As a result of this workshop, it was evident that ISAC and the members of the international cytometry community should immediately begin to engage with genomic cytometry and create an ecosystem to support those already in the field
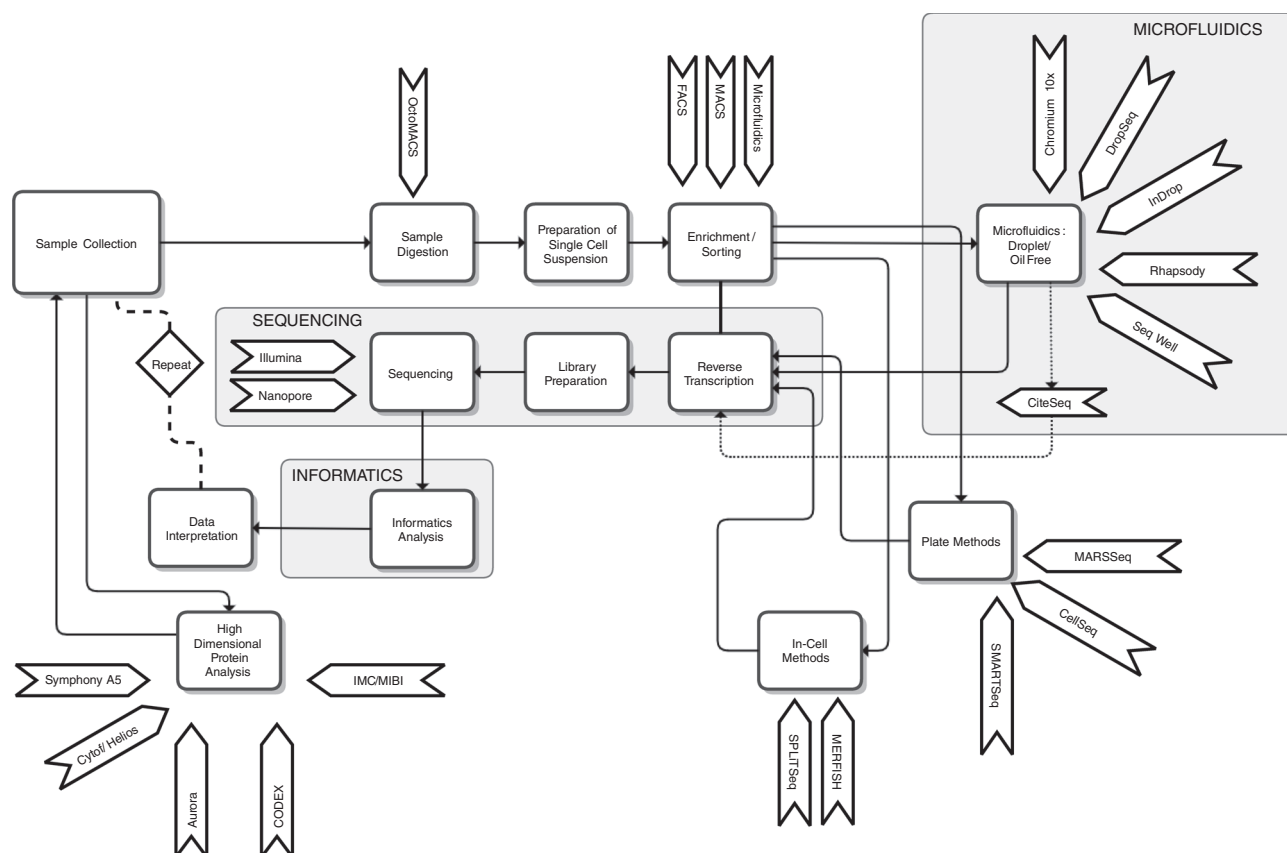
Figure 1. Outline of how the world of traditional cytometry (fluorescence, mass and imaging cytometry) interacts with the emerging field of genomic cytometry. These include, but are not limited to, expertise in cell preparation, cell sorting to isolate cells of interest, index sorting to match protein expression data to resulting sequencing data, and antibody staining methods.

and to bring new people into the field. There are many ways to do this, but the need for education, peer support, and training opportunities, as well as the formation of ISAC best practices in genomic cytometry were high on the list of requirements. The modern cytometrist must engage with these technologies and not allow their single-cell expertise to be lost or needlessly reinvented, helping to build an exciting future for single-cell science.

WS07: CYTO LAB HACKS: INSPIRING INNOVATION IN CYTOMETRY THROUGH OPEN COLLABORATION

Jakub Nedbal, Bunny Cotleur, Dominic Gagnon, Virginia Litwin, Jenny Molloy, Betsy Ohlsson-Wilhelm.

Introduction and Aims

Open Science is an active process of a cultural change, shifting the research conduct from competition toward collaboration and early dissemination. Open Science can be defined as "transparent and accessible knowledge that is shared and developed through collaborative networks" (19). The International Society for the Advancement of Cytometry (ISAC) has been an early champion of Open Science in the biological sciences by introducing the MiFlowCyt (20), FlowRepository (21), and the FCS data file standard (22,23). Here, we are proposing a new transparent platform for searching, disseminating, and collaborative sharing of cytometry innovations.

Cytometrists often generate innovations to protocols, instruments, methods, teaching materials, and products. Efficient dissemination of these cytometry innovations delivers

benefits to the innovator and the community (24,25). Yet there are no dedicated platforms to support sharing of cytometry innovations. Therefore, authors publish their innovations in redacted form as a part of research articles, post them on websites (institutional, blogs, social media) or do not disseminate them at all. Consequently, many cytometry innovations never reach their full potential by not being seen, used, or adapted by the community.

In this workshop, we launched CYTO Lab Hacks as an online free and open forum for sharing and collaborative development of cytometry innovation. A group of invested volunteers will build this forum from the ground up under the oversight of the ISAC CYTO Innovation Committee. CYTO Lab Hacks will be developed according to the principles outlined in the Global Open Science Hardware Roadmap (26). It will be resourcefully built on existing online platforms, which will be repurposed to create a cytometry-specific network of resources. The overarching aim will be to enable streamlined deposition and sharing of cytometry innovation under a common and widely recognizable brand. The title "CYTO Lab Hacks," used throughout this manuscript for consistency, remains tentative, and may still change in the future as discussed below.

Our workshop attracted a mixture of facilities managers, academic scientists, and industry representatives. We presented ideas and questions to the audience and processed their feedback and poll results. We gained the understanding of the needs, pains, experience level, and opportunities for the proposed innovation sharing forum. The workshop outcomes will help us guide the development of CYTO Lab Hacks. The aims are to accelerate and increase the impact of early stage cytometry innovation and to make ISAC the leader among the biological societies in the promotion and championing of free and open science hardware.

Methods

We used three channels to collect information prior to and during the workshop: a pre-workshop survey, workshop polling, and a 60-min moderated discussion with the audience. All three efforts were designed to understand the audience needs and to guide the future development of CYTO Lab Hacks. We gauged the existing level of awareness, usage patterns, licensing, and publishing opportunities for free and open source software and hardware. We aimed to learn the needs, pains, and opportunities in relation to searching for, sharing, and collaboratively developing cytometry innovations. The workshop included a presentation of examples of recent cytometry innovations.

A pre-workshop online survey (Forms, Google, Mountain View, CA) was repeatedly advertised prior to the conference on LinkedIn, Twitter, and multiple cytometry forums. We used a bespoke logo (Fig. 2A) and an advertising poster to capture the intended audience attention. The pre-workshop survey results are summarized in the supplementary note and Supplementary Information WS07_SI Figures S6–S13.

At the start of the workshop, the audience was polled for objections to audio recording, which was intended only

for use in writing this manuscript. Receiving no objections, the workshop audio was recorded using the available AV technology. The workshop was started with a 20-min motivational talk and 10-min question time with J. Molloy (Cambridge University, UK). An Internet link using Skype (Microsoft, Redmond, WA) and a USB boundary microphone (UB-1, Samson, Hicksville, NY) connected to the presentation computer facilitated this remote workshop contribution.

The rest of the workshop was structured as an open discussion of the panel (J. Nedbal, B. Cotleur, D. Gagnon) with the audience. The structure and timing was maintained through a slide presentation accompanied by live polling (Slido, Bratislava, Slovakia). The feedback from the audience was captured on the audio and through the live polling.

The workshop attracted 40–50 participants with ~20% contributing to the live poll.

Outcome

This section summarizes the content of the discussion and the resulting feedback. We covered the topics of mission statement, branding, identity, platform, structure, licensing, vendor engagement, and outreach.

The aims of CYTO Lab Hacks (Supplementary Information WS07_SI Fig. S1) listed in the descending level of gained support are 1. creating a sharing platform to boost the impact of cytometry innovations; 2. widening collaboration, engagement, and participation; 3. advocacy, education, and standards for sharing.

We assessed the branding proposal for CYTO Lab Hacks including the title, proposed logo (Fig. 2A, Supplementary Information WS07_SI Fig. S2) and other branding assets. We identified problems with the title "CYTO Lab Hacks," potentially devaluing the "CYTO" brand. Alternative title suggestions have been proposed (Supplementary Information WS07_SI Table 1) and an acceptable title will be selected shortly.

CYTO Lab Hacks should be organized by a task force overseen by the CYTO Innovation Committee (Supplementary Information WS07_SI Fig. S3). Actual "Lab Hacks" are to be demonstrated during CYTO Innovation and in the ISAC booth, to promote the initiative at the CYTO meetings (Supplementary Information WS07_SI Fig. S4). We proposed a visit to a local makerspace during CYTO 2019 to validate the potential of these community workshops to scientists. We also encouraged the audience to engage with institutional bioengineering departments, workshops, and local makerspaces.

Licensing, safety, and legal issues were identified as important considerations for CYTO Lab Hacks development. Careful management will be required to mitigate liability issues, manage intellectual property ownership, prevent endorsement of potentially unsafe practices, and protect vendor interests as CYTO Lab Hacks develop. The discussed strategies include limiting contributions to registered users, adopting procedures followed by established project-sharing platforms, and seeking legal advice.
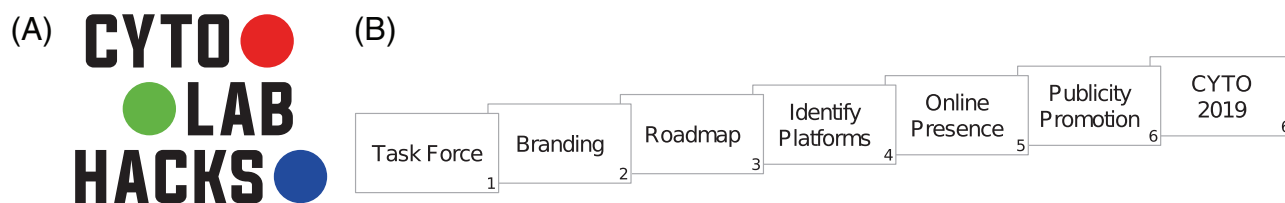
Figure 2. (A) Proposed logo for CYTO lab hacks features colorful bright dots representing cells, dot plots, and fluorophores, all common to cytometry. (B) Roadmap for the first year of the project. [Color figure can be viewed at wileyonlinelibrary.com]

Conclusions and Perspectives

Routinely cytometrists, as other scientists, develop innovations to methods, protocols, instruments, teaching materials, and products. These innovations deliver additional benefits to the innovator and the wider community when disseminated efficiently. They generate community feedback and spark follow-up collaboration and further innovation. Currently, there are no clear incentives or standardized platforms for sharing cytometry innovations. Consequently, cytometry innovation dissemination, sharing and collaboration is cumbersome, ineffective or slow; relying on traditional publishing or a variety of scattered online resources.

CYTO Lab Hacks community was established during the workshop to drive the development of a platform for searching and depositing cytometry innovations. The goal is to increase the innovation rate in cytometry by minimizing barriers in collaborative sharing of innovations according to open science principles (19,26). CYTO Lab Hacks will enable sharing regardless of and alongside peer-reviewed journal publications. Both the wider cytometry community and contributors will benefit from CYTO Lab Hacks. Searching for ideas and innovations will become centralized and thus more effective. Contributors depositing innovations will increase their impact through higher visibility, proliferation rate, community feedback, follow-up collaborations, and further innovations.

CYTO Lab Hacks is developing into a free, open, transparent community-run collaborative innovation forum within ISAC, revolving around an online platform. The development is taking place in a series of steps guided by the workshop outcomes and summarized in Figure 2B.

CYTO Lab Hacks has become an open group of volunteers. We are now working to establish efficient communication channels to create and retain the momentum within this group. We coordinate with the CYTO Innovation Committee to remain accountable and aligned with ISAC's needs, perspectives, and bylaws. We are developing CYTO Lab Hacks to adhere to and champion free and open science principles as outlined in the Global Open Science Hardware (26) guidelines. We plan to synergistically engage with other scientific communities sharing similar aims.

CYTO Lab Hacks will develop gradually. We will first redefine the CYTO Lab Hacks identity (Supplementary Information WS07_SI Table S1) to create an instantly recognizable brand. Then a roadmap for CYTO Lab Hacks will be developed to set goals and priorities over the following years (Fig. 2B). A range of online social media platforms, collaborative project management tools, data repositories, and open source project tools will be assessed to identify those best suited for CYTO Lab Hacks. The tools used by other science and maker communities will be analyzed to understand correct practices in licensing, communication, and management. Channels will be setup within suitable online platforms under a common branding umbrella. These platforms will be promoted for the use in the cytometry community to share emerging innovations. Presence and activities at CYTO 2019 will be developed. These may consist of a showcase of innovations, workshop, tutorial, and invitation of speakers championing open science.

We expect CYTO Lab Hacks to develop over the next years into a sustainable and instantly recognizable platform for cytometry innovation sharing. Its success will cement the position of ISAC as a leader in promotion of open science among the biological societies.

Acknowledgments

WS15: The Status and Future of Microbiome Flow Cytometry

Frederiek-Maarten Kerckhof, Peter Rubbens, Hyun-Dong Chang, Jakob Zimmermann, Ruben Props, Frederik Hammes, Susann Müller.

Introduction and Aims

Microbiomes, ranging from the human microbiome to man-made and natural ecosystems, perform essential services. For example, human microbiome dysbiosis can be indicative of the host's health status, while environmental microbiome changes may impact global biogeochemical cycling. This workshop set out 1. to raise awareness within the general ISAC/cytometry community of the emerging field of microbial community flow cytometry (MCFC) and 2. to initiate interaction with practitioners in the field of higher eukaryote (e.g. mammalian immune cells) flow cytometry. Whereas classical immunological flow cytometry aims to dissect the phenotypic diversity of cells of the same genotype, the unique challenge of MCFC is the vast diversity of different genotypes

present within a microbial community. Furthermore, even isogenic (biotechnological) microbial populations can exhibit a wide variety of phenotypes. Multiparametric phenotyping of immune cells (27) and interpretation of such data (28,29) have advanced the field of cytometry significantly in recent years. While there have been developments in the state-of-the-art phenotyping of microbial communities (30,31), this workshop also aimed at discussing how MCFC could benefit from the developments of higher eukaryote flow cytometry (29). Five topics for discussion were addressed at the workshop, which are outlined in the methods section below.

Methods

Workshop survey. A live survey was performed at the start of the workshop using slido.com to capture a general overview of the workshop participants' existing experience with MCFC (for questionnaire and responses, see Supplementary Information WS15_SI Table). A sign-up sheet was handed out as well for future communication, and anonymous questions could be asked using the slido.com interface.

Workshop methodology. Each of the five topics (as listed below) was introduced by a different workshop co-chair in a five-minute presentation. A ten-minute discussion with the audience ensued under the coordination of the workshop facilitators on predefined discussion points. All audio was recorded to enable accurate reporting and a list of participants' names, association, and email addresses were collected.

i. The set-up and purpose of MCFC assays, in addition to simple cell enumeration, was discussed with a focus on flow cytometric fingerprinting (30–32). Additional questions addressed possibilities to increase resolution for MCFC and the inclusion of appropriate experimental controls.

ii. With regards to the screening of microbiomes in human environments, a relatively novel application of MCFC (33), the discussion focused on sample preparation of, for example, fecal slurry and, more generally, MCFC of any type of particulate-bound microbes or aggregated microbes (e.g., biofilms). Viability staining of (strict) anaerobes was put on the agenda as well.

iii. Complementarity of MCFC with other "omics"-approaches that are the current state of the art in microbial community characterization (e.g. marker gene amplicon sequencing (34,35) or even single-cell-omics (36)) was a major point of discussion as well. Here, the topic was focused on how subpopulations (or single cells) should be sorted considering experimental design and downstream analysis.

iv. Regarding the link between of MCFC fingerprints and ecological metrics (37), the synthesis of fingerprints into interpretable ecological metrics was put on the list of discussion points.

v. Finally, with respect to data analysis, it was shown that there are unique computational challenges in MCFC: in part due to flow cytometry automation (38), MCFC data sets are ever increasing in size and complexity. Furthermore,

the inclusion of possible spatiotemporal structuring in microbial communities and the incorporation of domain knowledge within synthetic ecology and biotechnological applications remains challenging. While many robust and efficient packages for fingerprinting and automated flow cytometry data analysis exist in the field of higher eukaryote flow cytometry, data analysis in MCFC remains a major bottleneck. Therefore, another goal of the workshop was to introduce MCFC data to cytometry bioinformaticians.

Results/Outcome

The workshop had 56 attendees, from whom we recorded between 35 and 39 responses for each question on our slido.com interactive survey (see Supplementary Information WS15_SI Table). Thirty three percent of responders had never analyzed microbial communities using flow cytometry. Sixty one percent of responders considered microbial flow cytometry data acquisition "not without challenges, but feasible," whereas 28% considered it to be "very challenging." Interpretation and analysis of microbial flow cytometry data was considered "not without challenges, but feasible" by 47% of the responders, "very challenging" by 42%, and even "nigh impossible" by 3% of the responders, clearly indicating the need for more attention toward data analysis and interpretation in the field of microbial flow cytometry. Nevertheless, when screening Flow Repository (21) on 28th of April 2018, we found that roughly 5% of all publicly available FCS files contain microbial data. This not only proves the contribution of the microbial community flow cytometrists to the wider flow cytometry community but also highlights the existing room for growth in the MCFC field.

During the live discussions, we identified that some subtopics of the list we put forward (see Methods section) elicited deeper discussions: 1. Sample preparation, especially of "clumped" or aggregated cells in biofilms or on particulate matter requires new protocols with appropriate controls. 2. Fixation and stabilization can greatly enhance the applicability of microbial flow cytometry but cannot be used for viability assays. 3. There is a need to assess, which (viability/physiology) dyes (or dye combinations) are suitable for non-fixed "natural" communities, and further investigation into the benefits of combining dyes is warranted. 4. It needs to be clarified how anaerobic bacteria and communities (even with fixation) can be analyzed. 5. Finally, additional validation of the linkage between flow cytometric fingerprints and microbial diversity is welcome. In particular, methods currently under development to infer the composition of a microbial community from fingerprints obtained from cultures of defined species/strains should yield great benefits.

Discussion

In the discussion below, we explore highlights from the workshop, focusing on questions and comments from participants.

Set-up and purpose of MCFC assays. Four main topics were addressed: 1. dealing with aggregated cells, 2. instrument variability, 3. fixation, and 4. enhancement of resolution for MCFC assays (which could improve fingerprinting as well).

With respect to aggregated cells, the discussion focused on the dispersal of cells associated with particles (e.g. fecal samples) and on coaggregated cells (biofilms/doublets/triplets). It was pointed out that several published dispersal protocols exist (see section 5.3 in (39) for an overview). For example, predilution and sequential filtering over 30-µm and 5-µm filters is recommended for fecal material preparation (e.g. for the application of MCFC fingerprinting on the stool of mice to infer the health status of the host (33)). Ultimately, regarding aggregated cell dispersal, sonication is the preferred method of choice, although it was found that a straightforward Tween treatment could be effective in specific environments as well (e.g. nose swabs). It was suggested that enzymatic methods for aggregate disruption would potentially introduce too much bias when working with microbial communities. Microscopy should be used to evaluate the efficacy of aggregate dispersal. Optical scattering resolution in current flow cytometers is generally too limited to distinguish single bacterial cells from doublets or triplets in microbial communities where cells of different sizes are present, although technological advancements are trying to resolve this (e.g. small particle detection modules, violet or near-UV scattering and nano-flow cytometry of extracellular vesicles (40)). Protocols to isolate single cells from most matrices and environments do exist. However, methods including cell washing inherently lead to cell loss.

Inter-instrument variability and the type of staining could have a strong impact on diversity estimation by MCFC fingerprinting. Whereas inter-instrument variability is difficult to control, some attention was brought in the discussion toward the ongoing search for dyes (or combinations of dyes) that are usable for physiological characterization (e.g. for membrane integrity) without fixation.

Regarding stabilization and fixation several published protocols are available (41,42), but they encompass loss of cells (due to washing steps) and do not allow viability analysis.

Finally, regarding the increase of (fingerprinting) resolution, the question was raised to what extent fluorescently labeled antibodies could be used to enhance resolution and detect specific taxa, as is done for specific cell types in mammalian flow cytometry. The main limitation so far is that the available antibodies are mostly developed against pathogens. However, work is underway to make a monoclonal library using IgA (natural) antibodies against gut microbiome bacteria (see section below). These can subsequently be used to sort and enrich specific subpopulations using FACS or MACS for downstream analyses. As an alternative to antibody labeling, Flow-FISH (fluorescent in-situ hybridization) (43) can be used; however, the protocol is tedious and often only limited 16S rRNA sequence information is available to design specific probes.

Screening microbiomes in human environments. Using MCFC, the mammalian intestinal microbiota has been profiled and disease-associated dysbiosis states have been diagnosed (33). Microbiomes of gnotobiotic animals with defined diversity can be leveraged to enable the determination of composition by flow cytometric fingerprinting. In this context, cell-permeant dyes (e.g. staining DNA) can enable sorting of viable pure bacterial species. The viability of cells, in particular (strictly) anaerobic organisms, sorted from the microbiota in human (and other mammalian host) environments was an important topic of discussion. Some possible protective strategies were suggested, such as sparging the sheath liquid or pressurizing the flow sorter with nitrogen or argon. Adding reducing agents may also circumvent the need to place the cytometer into an anaerobic hood. The question whether viability staining can be linked to cultivability was raised. Published evidence suggests this is not always the case (44). Finally, flow cytometric fingerprinting of microbial communities originating from other body sites than the gut was discussed. In the case of skin, low microbial recovery has been observed. However, it is important to note that low cell numbers can lead to a high variability of the population diversity estimates (45), which shows the necessity of thorough sampling and recovery strategies in the case of human microbiome flow cytometry.

Complementarity of MCFC with other "Omics"-approaches. It was suggested during the discussion that quantitative PCR and sequencing could give the same results as MCFC and fingerprinting. Whereas indeed it has been shown that MCFC diversity estimates (even with a single nucleic acid dye) can be linked strongly with amplicon sequencing estimates (31), an important motivator for using MCFC is its speed and relative ease of use as opposed to PCR-based and sequencing-based approaches (with possible primer bias). This was already shown for freshwater samples (31), but further validation in other environments (e.g. host-microbiome) will be necessary. It was also discussed that the joint use of MCFC and amplicon sequencing can be used to make compositional data representative of absolute taxon abundances (34). Briefly, the question was raised about sorting and massively cultivating the sorted cells on a wide array of media and growth conditions. Although these culturomics of isogenic cultures hold value, typically phenotypes in an isogenic population (e.g., in single colonies on agar plates) are very different from the phenotypes obtained in co-cultures, due to emergent phenomena and interactions. Ultimately, the increase of throughput of index-based sorting and single-cell genomics (36) was found to be promising as an ultimate validation of MCFC diversity estimation.

Ecological metrics derived from flow cytometric fingerprints. The relationship between ecological stability and functionality of biotechnological microbial systems has been established, for example, biogas production (46). Therefore, it was discussed how MCFC-based ecological stability metrics could be determined. From the discussion, it became clear that a unified approach to infer ecological parameters from flow cytometric fingerprints should be based on mathematically sound distribution-based methods for phenotypes, as used in

macro-ecology (47). Nevertheless, even in the macro-ecology literature, consensus is not always reached (48).

Computational challenges for cytometric fingerprinting. It was discussed that finding multicolor dye combinations (as discussed in the setup & purpose of MCFC section) should increase the phylogenetic resolution of FC fingerprinting. Also, the instrument detector quality (particularly for the light scatter signals) was brought up as an important factor for single-cell identification, as previously described (49). A question was raised whether microbial interactions influence phenotypes, which is something several of the panel members are currently evaluating. As machine-learning algorithms always involve a learning stage with axenic communities, it is hard to cope with emergent phenomena such as phenotypic changes that occur due to interactions. An alternative solution could be to use co-occurrence networks like the ones used for sequencing data (50).

Conclusion & Perspectives

The workshop was marked by high participation and lively interaction, which indicates that the aim of the workshop to create awareness and interaction with non-microbial cytometrists was successful (see also Supplementary Information WS15_SI Table). It was clear that standardization of wet-lab procedures is essential for comparability of MCFC data. These protocols need to be disseminated in an intelligible manner with rigorous detail (e.g. (42)). While many solutions exist for several issues (e.g. fixation), these are rarely consistently used among different research groups. The development of pretreatment, staining, and analysis procedures that work over a range of different organisms and environments will be essential for a widespread application of microbial flow cytometry. Furthermore, there are still unexplored opportunities for bioinformaticians in the field of FC of higher organisms to apply their algorithms and approaches to MCFC data. Current data-analysis methods for MCFC are not mature, and development of tailored multivariate statistical approaches for MCFC is required. In order to enhance the interaction within the ISAC community and the worldwide MCFC community as a whole, we will start an MCFC mailing list where people can ask questions and exchange ideas and protocols. For furthering the data analysis field, a GitHub community will be initiated where we will organize all publicly available software and initiate a data challenge in the near future (https://github.com/MCFC-Community).

CHAPTER 1: TRENDS CONCLUSION

The workshops summarized in this chapter produced several concrete outcomes, such as: a. reviewing the state of the art in selected subfields; b. raising awareness of certain problems or practices; and c. seeking input from the community on actions to be taken. The formation of the CYTO Lab Hacks group, for example, is a direct result of WS07, as are plans for engagement and collaborations among different cytometry communities as well as between cytometry and genomics groups.

To conclude, live discussions such as those at the workshops, are a way to survey knowledge and attitudes across members, exchange ideas, build consensus, and formulate plans for moving the entire society forward.

Where is cytometry going? What are the trends in this field in 2018? Increasing detail of biological studies, demands for faster sample processing, and more accurate data analysis drive technological advancements in cytometry. Mass or spectral cytometers and accompanied them methodologies allow for more accurate detection of increasing parameters number in samples. Genomic cytometry permits researchers to gain insight into unique single cell characteristics. All solutions enable fast and unbiased large and complex data analysis. All these however can be only possible if a frequent and open interdisciplinary communication between researchers and technologists, instruments and reagents manufacturers, service providers, and end users happen. Dialogue between various members of cytometric community can be facilitated by thematic groups formation and thanks to internet technology discussions may be conducted online and knowledge data base can constantly and interactively grow and stay available for research leading to further innovation.

Cytometry can advance if both technology and application co-evolve—one driven by the other.

CHAPTER 2: SHARED RESOURCE LABORATORY (SRL) BEST PRACTICES

Four workshops presented within the SRL track at CYTO 2018 address several areas that can present challenges within the operations of an SRL. WS01 and WS05 deal with generalized operational practices within an SRL, while the WS02 and WS10 deal with challenges associated with specific high-end technologies often found in the SRL.

WS01, Balancing Science and Service in an SRL, was designed to address the dual role of SRLs in providing both scientific innovation as well as access to expertise and instrumentation that has been properly maintained and qualified. While most SRL directors, managers, and staff realized the importance of both of these roles, finding ways to balance these complementary but often competing roles was the main focus of discussion in this workshop.

WS02, A Successful Imaging Flow Cytometry Facility: Guidelines for Management and Publication set the goal to generate a consensus on "Best Practices" for the implementation, usage, and publication of data generated by the powerful technology of Imaging Flow Cytometry (IFC) in an SRL environment. In discussion groups moderated by the workshop organizers, ideas were generated which supported increased utilization of this technology as well as ideas on how to establish publication standards for the reporting of data generated using IFC.

WS05, Identification of Areas where Software Tools can Contribute to the Successful Operation of a SRL, dealt with the assessment of what software tools are currently available (or needed) that can aid in the successful operation of an SRL. This workshop assessed existing tools and how they can

be improved, as well as discussed what tools still need to be developed. The pre-workshop survey also provided the beginnings of a repository of software solutions specifically targeted for SRLs, which could be built upon and curated by the SRL community going forward.

Finally, WS10, Best Practices for Development and Implementation of a CyTOF Core, focused on identifying the difficulties in implementing and maintaining the CyTOF (mass cytometry) technology platform that are limiting its widespread adoption. Through active audience participation and discussion many ideas and best practices were generated and are presented in this report. The key findings are summarized, as well as best practices for new and existing CyTOF cores proposed.

WS01: Balancing Science and Service in a Shared Resource Laboratory (SRL)

Joanne Lannigan, Rui Gardner.

Introduction and Aims

The mission of SRLs is to provide key technological services to researchers/investigators, as well as provide guidance and expertise on the use of such services in scientific investigations (51). A major role of SRLs is to also insure quality data, which is reproducible (51,52). However, another very important role of the SRL is to be on the forefront of new technologies and their applications, as well as the development of new applications and tools to enhance an investigator's resources for discovery (51,53,54). While one may think these roles should be complimentary, often due to time, staff, and other resource limitations, they may be competitive and in conflict.

This workshop sought to address some key questions that are associated with this dual role. First, in order to better justify the need for this dual role, one must first identify the benefits as well as the potential conflicts associated with providing both science and service activities within a core environment. Second, a necessary requirement for the engagement in both science and service activities is the need for resources to support both. While the provision of service is commonly funded through the use of a fee-for-service model, identifying resources to support scientific endeavors can be a bit more challenging. Should this be a component of the fees charged for service or should there be a separate source of support? Finally, assuming an SRL does support both science and service, how are these two missions balanced within the overall operation? Should everyone be involved in each component, or should there be dedicated individuals to each component? How would an SRL allocate time and priorities for these activities? Should service be a first priority over science, or should both activities be considered a top priority? This document is a summary of the survey results and the active discussions by the participants in the workshop.

Methods

In order to assess the current status of the SRL community on the dual role of science and service, the workshop organizers (Lannigan, Gardner) solicited input from the SRL community through a Google survey issued prior to the workshop (Supplementary Information WS01_SI1). The survey was shared with the community through the Purdue Cytometry List, the Cytometry Community on Google+, Flow Cytometry Researchers Group (LinkedIn), ISAC's LinkedIn page, the Australasian Cytometry Society mailing list, and various twitter accounts. The results were tabulated, summarized, and presented at the CYTO 2018 workshop. The survey results and workshop presentation can be found in the Supplementary Information. The ~50 workshop participants were then divided into three groups; each group assigned a specific question to discuss. Each group was asked to identify a spokesperson for the group who was charged with taking notes from the group's discussion and presenting them to all the workshop participants. The following discussion questions were assigned:

Group 1: Discuss the pro's and con's of an SRL performing both service and science. Do not attempt to decide which scenario is better, but rather just identify the benefits and penalties that you would associate with each scenario.

Group 2: If an SRL was to perform both science and service, where would/should the resources for the science part come from? Who would pay for the component of staff salaries associated with the science/research activities?

Group 3: How can an SRL balance the need for both science and service? Should everyone be involved in science and service or should there be dedicated individuals for each? How would you allocate time and priorities for each of these activities?

Survey Results

From the survey responses, the clear majority of SRLs (69.9% of 113 respondents) incorporate science or some type of R&D activities in their facilities, versus 30.1% that responded that they do not have adequate institutional support or enough staff time (see Table 2 or Supplementary Information WS01_SI3 for full survey results). From the latter, half believe it does not impact their facility negatively and 55.9% do not have intentions of implementing R&D activities in the future. In contrast, the large majority (89.9%) of those who currently implement some type of R&D, find it benefit for both SRL staff and users and the institution overall, whereas only one respondent found it did not. This agreed with the discussion during the workshop. Regarding the source of resources required to perform R&D, 70.9% reported that funding came from their operational budgets. (Table 2). In addition, some SRLs take advantage of beta testing of instruments and reagents that are provided at reduced or no costs, which can be included in the 46.9% of SRLs that use collaborations to support at least some of their R&D activities. Finally, some institutions provide internal funding sources, though rarely, for independent research within the SRL (from survey, 25.3% are supported by internal or external grants). Only 12.7% of survey respondents reported to obtain direct

Table 2. Summary of 113 responses to survey

QUESTION	RESPONSES
Does your facility incorporate any research or developmental activities into the normal core operations?	Yes - 69.9%
	No - 30.1%
If you answered No, why not?	No support - 67.6%
	No enough staff time - 58.8%
	No staff interest - 5.9%
Are there plans to do so in the near future?	Yes - 44.1%
	No - 55.9%
Do you believe this negatively impacts your facility in any way?	Yes - 50%
	No - 50%
If you answered yes, what % of overall facility hours are dedicated to R&D?	1–5% - 39.2%
	6–10% - 26.6%
	11–15% - 11.4%
	>15% - 22.8%
Are there dedicated people that perform this function for the facility?	Yes, one or more dedicated staff - 19%
	No, everyone participates - 51.9%
	No, a selected few participate - 29.1%
Have these activities benefited users/institution/core?	Yes - 89.9%
	No - 1.3%
	I do not know - 8.9%
Who do you think benefited?	Mostly users - 17.8%
	Core staff - 5.5%
	Institution - 2.7%
	All of the above - 74%
Where do resources come from to support these activities?	Grants - 25.3%
	Operational budgets - 70.9%
	Institutional support - 12.7%
	Collaborations - 46.9%
How do you allocate time and priorities for doing science and service?	As time permits – 75.3%
	Scheduled protected time- 19.5%
	Other- 5.2%

institutional support outside of the operational budget (Table 2).

From the survey responses, we found that 19% have one or more dedicated staff for R&D activities. When it comes to time allocated to science, the survey interestingly reported 22.8% dedicate over 15% of the SRL's staff time, and about 65% dedicate 10% or less.

Results Workshop Discussions

We should begin by noting that the discussions that took place during the workshop may have been biased by the fact that the participants that attended did so because of an interest in learning about the balance between science and service. However, the sentiments that were heard were similar to what we found in the responses to the survey, regardless of the group surveyed. The majority of those who attended the workshop either managed or worked in an SRL. Some comments that resulted from the survey that indicated a lack of clarity to the definition of "science" in this context led the organizers to provide the workshop participants with a more clearly defined idea of what we were referring to as science for the purposes of the discussions. This was defined in the presentation that was presented at the workshop and was broadly defined as both R&D type of science, as well as collaborative science. This clarification did not seem to change the overall sentiments and positions of the workshop participants when compared to the respondents of the survey.

From the workshop discussions, there was a clear consensus that service needs to be prioritized in an SRL. However, it was also agreed, by workshop participants and the majority of survey respondents, that incorporating science in the facility brings advantages to the institution, ranging from staff development and new methodology implementation to improvements in quality of research of those who use the SRL. To balance R&D in the core facility, the general consensus from workshop participants is that everyone in the facility should be involved at some level with scientific activities, though there was no clear objection that some staff could be solely dedicated. The following summarizes the main discussion points from the workshop.

Pros and Cons

R&D activities in an SRL can be both beneficial and detrimental to users and a balance needs to be struck. Having clear directives from administration regarding sources of

financial support and time dedicated to R&D would help in establishing good practices for the implementation of these activities as well as in developing tangible metrics to improve this balance. Participants mentioned it keeps SRL staff and users, as well as the institution, on the forefront of the field and technologies, establishes SRLs as experts and increases reputation, helps in recruitment of faculty to the institution, helps in recruitment and retention of creative staff, opens career path options, and potentially contribute to successful funding opportunities. On the opposing side, it was also pointed out that these activities consume resources (time and money), can detract attention away from users, and the science performed may be mediocre due to resource constraints. The group also felt that often results of these activities may not be tangible and therefore hard to evaluate their benefits.

Where Should Resources Come from?

Workshop participants stated that the majority use their core's budget, in agreement with 70.9% of survey respondents who indicated that R&D was supported by their operational budget. Collaborations with users should be encouraged as they benefit directly the institution, distribute resource expenditure, and make it more of a tangible benefit. One workshop group felt that, even with funding sources, the demands of a core generally overwhelms any intended research.

Balancing Science and Service

Implementing and executing R&D activities in the SRL is not an easy task. One cannot ignore some of the less enthusiastic comments (see Supplementary Information WS01_SI3) that display a sentiment of distrust and frustration toward the lack of support from the institution and investigators to bring science into the SRL. During the workshop, some participants suggested that one could create the right environment to incorporate R&D in the core facility by providing an excellent service and training users to self-operate instrumentation. This will allow utilization of free time during long experiments that require little intervention and monitoring (e.g., during long sorts).

Value of R&D in Staff Development and Faculty Recruitment/Retention

Workshop participants also felt that having the opportunity to be involved in R&D activities in their facilities is extremely helpful for staff development and job satisfaction. While it was acknowledged that not everyone is interested in these types of activities, those who are may seek positions elsewhere where such opportunities do exist. Also, of note, was the importance these activities provide in terms of key faculty recruitments/retentions, as it is more likely for the facility to be perceived at the forefront of the technology and a resource to the investigator's own research. Whether these research activities took place seemed to be more dependent on the Core Director/Manager's interest to implement such activities rather than on institutional support. However, there seemed to be some consensus, that at least at a minimum, the institution's overall culture in supporting such activities,

whether financially or in concept, played a major role in whether or not these activities happened.

Conclusion

The survey results and these discussions do not provide all the answers, but hopefully will provide some insight to the cytometry community at large regarding the prospective dual mission of SRLs in the future. While the survey and workshop participants were from the Cytometry community (both imaging and flow), these views are likely to be similar for other SRLs as well. This was evidenced by a similar survey conducted by the Mid Atlantic chapter of the Association of Biomolecular Facilities (ABRF), where the results were quite similar to those found within the Cytometry community. Recognizing the importance, as well as the constraints, of providing both science and service is a first step in finding the right balance. While some SRLs may have more resources to accomplish this dual role than others, most SRLs will need to find ways to support this endeavor going forward.

WS02: A Successful Imaging Flow Cytometry Facility: Guidelines for Management and Publication

Dominic C. Jenner, Aja Rieger, Andy Filby, Ziv Portat.

Introduction

Since the commercial release of the first platform in 2005 by the Amnis Corporation, Imaging Flow Cytometry (IFC) has rapidly evolved into a powerful discipline that has enabled researchers to ask unique and fundamental questions about biology at the single cell level. For those of us that have been involved with the technology since the beginning, it was and still remains a challenging technology to get the very best from, especially in the SRL/flow core environment where it can often require far more attention and input than conventional flow cytometers (analyzer and even cell sorters). In many cases the first challenge faced is how to secure funds to purchase a system that in many cases requires more consideration about how it will be utilized and what critical biological questions it will be used to address. Once this obstacle is traversed and the system is purchased, this is often where the real challenges begin. The next challenge is being able to convince users that the technology can provide them with novel/better/more advanced data that simply using classical flow or microscopy alone or together. The first qualifying questions should be "does the question you want to ask of your cells absolutely require or would benefit from morphological/spatial information"? "Will it benefit from

collecting spatially registered controlled and quantifiable images?" "Do you need statistically relevant information from collecting 100,000 to millions of cells"? If the answer is "Yes" to any or all of these questions then one can say that the experimental need for IFC is there. The next challenge however is local expertise. To get the best from IFC, it is essential that there is highly trained staff that can maintain the system and help design/acquire the experiments. The SRL/flow core is classically the perfect environment for this development and retention of expertise; however, SRL/flow core staff often has many other demands and IFC can be very demanding on staff time. Moreover, IFC data analysis is much more challenging and involved due to the fact that it requires knowledge of image analysis techniques. Related to this, expert staff need to be retained and one way that this can be done is through reward for their expertise in IFC, possibly through named acknowledgement or authorship on papers where they have made significant intellectual contributions. The aim of this workshop was to bring together the IFC community to discuss how systems are managed within SRL and flow core laboratories and if there should be guidance published to assist in the best practices to publish IFC data sets. While there are currently guidelines for the publications of standard flow cytometry data and on the best practices for running an SRL laboratory (52), there are no specific guidelines for the publication of IFC data or how best to run one within an SRL, an issue that has been highlighted and discussed previously by Filby and Davies (55). The workshop was aimed at any scientist, SRL staff, and flow core staff that has an active involvement with imaging flow cytometry equipment within their institutes. The expected outcome of the workshop was to come away with a better understanding of how other SRL, and flow cores are building and maintaining their IFC user base, as well as the information required to put together guidelines on best practices for the management of IFC equipment and publication of IFC data sets.

Methods

The issues surrounding the best practice for managing IFC equipment and publishing IFC data sets have been talked about for a number of years. For this reason, at this workshop there was no pre-workshop survey issued, although people were encouraged to post open questions on the google plus forum (https://plus.google.com/communities/113171820758206750678) to be answered at the workshop (time permitting). After an introduction to the workshop and its goals, attendees were split into four groups. Each group was given a question/scenario with guiding questions to discuss. This was moderated by one of the four workshop organizers. Please note that the questions were for guiding purposes only, designed to induce discussion, some groups did not discuss all guiding questions. Each theme has a scenario-based question and a direct discussion-based question, designed to get people to discuss the issues in different ways.

Section 1: Scenario-based question relating to running an IFC in a flow core or SRL.

A researcher contacts the flow core for information about using the equipment. When you speak with the researcher you quickly realize that IFC would be perfect for their application. However, you get push back from the principle investigator (PI) on using IFC.

1. How can you utilize your knowledge to show IFC is the best option?
2. What steps can you take to show the PI what IFC can do?
3. How do you demonstrate the advantages of IFC over conventional flow cytometry or microscopy?
4. Can you give examples of projects that were not possible using conventional flow cytometry or microscopy?

Section 2: Discussion-based questions relating to running and IFC in a flow core or SRL.

Running IFC equipment in a flow core or SRL can be challenging. In your group please discuss the following questions using your own experiences from your facility.

1. How to recruit users to your IFC platform?
2. How do you maintain/follow-up on users?
3. How do you train staff/users to run samples?
4. How do you train staff/users to analyze their data?
5. What QC and maintenance do you use for the instrument (ASSIST / Rainbow beads, additional cleaning procedures)?

Section 3: Scenario-based question relating to publishing IFC data sets.

The IFC data acquired in your SRL is now being utilized in a publication; however despite being integral to the collection and analysis of the data, your comments on how best to publish the data are not being recognized.

1. What should be the contribution of SRL staff to the writing of an article (preparing figures, writing methods, participation in writing results/discussion)?
2. How is the scientific contribution determined—acknowledgement/authorship (and order of authors)?
3. How does your facility deal with disagreements over authorship in publications?
4. Do you think a set of published guidelines would help convince researchers to include all the relevant information need to both evaluate and reproduce the work?

Section 4: Discussion-based questions relating to publishing IFC data sets.

The publication of IFC data is becoming more and more common in the research environment. In 2012 Filby et al. published a communication to the editor regarding the standardization of publication regarding IFC-derived data (55). As an outcome of this workshop, we wish to produce a set of standardized, consensus guidelines for the publication of IFC data.

1. What would be the minimum set of instrument parameters you would expect to see in a paper publishing IFC data? Would you expect a difference between a methods

type IFC paper (90% IFC content) over a research type paper (20% IFC content)?

2. In your opinion, what is the best way to display mask/feature nomenclature in publications?

3. Should there be a standardized way to display IFC imagery in publications?

4. How do you best show mask development within a publication?

After 25 min, each group was switched for a different question/scenario. Once the workshop had finished a google form link with the workshop questions was posted to the IFC google plus forum for people who could not attend to fill in, there were three responses to this survey.

Feedback surveys were also handed out to all participants for the completion to gain an understanding of how well the workshop was perceived, data from these feedback surveys is available in the Supplementary Information WS02_SI Feedback_Survey_Results.

Results

Discussion notes were written up and shared with all workshop organizers (see Supplementary Information WS02_SI Discussion_Write_Up). The section described above fall into two distinct themes: management of IFC equipment in SRL or flow cytometry core facilities and discussions around guidelines for the publication of IFC data sets.

Theme 1: How to build a successful IFC facility. *Section 1*: Discussions were focused on how to increase peoples understanding of IFC and how it works and what it can offer. The main issue SRL/flow core staff voiced is a lack of understanding by researchers/users/PIs of IFC and its applications. This leads to comments like "why would I use that?", "We have always done it this way," and "why is that better than what I am doing now". Therefore challenging this mentality is key to recruitment of IFC users. Discussions focused on how to try and persuade people to try IFC as part of an experiment. These included giving interested users a simple explanation of the technology and/or performing a demo of IFC with sample cells to show the advantages of the system; working with unconvinced PIs to show them how the technology can help (specifically) their research and try to convince them to perform IFC experiments alongside conventional experiment; having preconsultations with researchers to discuss new technologies and how they work can help bring in new users that are interested. It was commented that once you have a researcher that has performed a successful experiment or gotten a publication with IFC data it can jump start an interest in the IFC and make the technology more attractive to other groups within the institute.

Section 2: Recruitment and maintenance of an IFC user base can be a challenge in an SRL/flow core. Many attending SRL members felt that the best way to recruit users was by having direct contact with the users, talking with them, and showing them the benefits of IFC over other techniques. SRL/core staff could also reach a wider audience by having special demos or seminars on IFC. Retaining users is also important and this is more likely to occur if user experiments "work," therefore making sure users have a good understanding of how to prepare samples for IFC and how to get the most out of the machine is important. Many agreed that the training of individual users was not too challenging and many adopted the "see one- do one-teach-one" approach. It was widely agreed that analysis of IFC data is by far the most challenging aspect of running an IFC facility. Again many SRLs/flow cores give basic training or offer to perform/check analysis for users. However, there is no substitute for experience and having a dedicated super IFC user/staff member is a good proposition for IFC data analysis.

All of the above assumes that the SRL/flow core has a good amount of experience in performing and analyzing IFC experiments, which if IFC is new to the facility may not be true. In this case, building up a facility specialist is a good way to help increase expertise within the SRL/flow core. IFC modules within flow cytometry training courses are becoming more common, and the manufacturer offers on-site training within institutes or advanced training at their Seattle offices. The IFC community is becoming more active in promoting good practice and other SRL's with experience might be willing to offer assistance to less experienced cores.

Theme 2: Should there be published guidelines for how best to present IFC datasets. *Section 3*: Publishing data in any environment can be a challenge but it can be particularly challenging for the SRL and flow core community who can sometimes be seen as a service and not part of the research process. Discussions focused on what credit should be given when helping with manuscript publication and how disputes can be settled between research groups and SRL/flow cores. Many agreed that the earlier publications are discussed the better chance there is of getting a name on a publication as SRL/flow core staff. Engaging with the researchers to find out how much help they will require for a particular set of experiments is also encouraged, as there is a big difference between helping to acquire the data and then being expected to perform the analysis and produce a data set for publication—this can increase the work load significantly. This topic falls into the authorship by contribution for determining named authors, if you have had influence over experimental and panel design, analysis techniques, and manuscript write up then authorship should not be debatable. In most cases SRL staff/flow core staff asked as a minimum for an acknowledgement within the paper for their facility. Where disputes do occur between research groups and the SRL/flow core it was said that it is largely up to each party to settle their differences (formal dispute resolution processes differ by institute), many try to introduce an unrelated third party to help with solving disputes. Many said it was hard as an SRL or flow core to truly enforce anything making some disputes difficult to resolve. It was generally agreed that publishing within a SRL/flow core was important to increase the reputation of the facility.

Section 4: The publication of IFC data sets is becoming more and more common in the scientific community; this is a great way to promote IFC and its advantages/disadvantages over conventional flow cytometry. However, there is still no guidance on how best to prepare IFC data sets for publication. The general consensus from the IFC community is that guidelines for publication would be beneficial; however, there were some individuals that voiced concerns over strict publishing criteria. These individuals felt that having strict guidelines would stifle the author's ability to present their data and methods in the ways they would like. For this reason, it would be likely that any guidelines published would be in the form of a set of recommendations rather than strict enforceable rules. Discussions were also had on what these recommended criteria should be (see Table 3 for examples, please note this is not a comprehensive list but some general thoughts from the workshop). The general consensus was that the more information provided within the paper (even Supplementary Information), the easier it is to replicate an experiment. IFC analysis works on the basis of features and masks, and

discussions were had about the best way to present this in IFC papers. The main consensus was that clearly written explanations of the features used was key to understanding the author's analysis process along with the visual examples of the masking strategy under taken as part of the analysis. The probable outcome of this discussion would be some additions to the MIFlowCyt guidelines as already discussed by Filby and Davies (55) (see Table 3). These would include some information on masks used and how features were selected.

Discussion

Imaging flow cytometry is a maturing field with a substantial and growing user base. However, in some circumstances, SRLs/flow cores still struggle to establish, maintain, and grow a user base and build expertise in IFC to help users with experimental design, data acquisition, and most importantly data analysis. Due to this, underutilisation of IFC equipment is a common issue in some SRL/flow core laboratories. Among the reasons for that are lack of image analysis training of flow core staff leading to insufficient assistance in data

Table 3. Summary of workshop discussions on IFC publication guidelines. All IFC experiments and analysis are complex and require an understanding of the collection and analysis methods. This table gives a summary of the ideas the workshop attendees had regarding publications of IFC data sets

IFC PARAMETER	PURPOSE	EXAMPLE
Data acquisition details		
Instrument details and set-up	Give the ability to compare the instrument used and to adapt the method to their system	ImageStream × MkII, Dual Camera, 405, 488, 647 nm lasers, lens used, EDF mode (when relevant), acquisition speed.
Channels collected including fluorophores used	Indicates what channels were utilized and which fluorophore the authors are viewing in each channel	Channel 02 (FITC), Channel 06 (Side scatter), Channel 07 (DAPI), and Channel 11 (AF647)
Acquisition parameters and number of events collected	Indicate how the cells that have been analyzed were acquired. A full breakdown of the acquisition graphs could be provided in supporting information if though necessary	10,000 in focus (Gradient_RMS_Ch01 $\geq$ 50) single cells (Area_M01 vs Aspect Ratio_M01) were collected
Data analysis details		
Masking strategy	It was thought that a comprehensive record of the masking strategy should be included in IFC data sets with a rational for how the masks were chosen and why. Images of masks (and rejected mask) can help to illustrate this point quite easily.	
	Masking nomenclature was also discussed. It was generally agreed that as long as a clear definition of all masks and the original mask nomenclature (e.g. Area_M01_ (Ch01)) was included within the methods section, shortened versions could be used for graph axes.	
Feature selection	A clear explanation for any specific features should be included within the methods section, especially those features that are not commonly used. A full description of feature and mask along with the original nomenclature (e.g. Intensity_Object_M02 (Ch02)) should be included in the any publication.	
Data presentation	Data should be presented clearly and precisely with a clear link between the data and the feature and mask used to derive the data set.	
Controls and gating strategy	A clear and detailed description of controls used should be provided, to illustrate and justify how the gates were chosen (As FMOs, 2nd antibody only, biological controls, etc.)	

analysis, limited awareness of the technology or inconsistent support. To aid in this, one part of our workshop was to openly discuss the way people recruit and maintain a functional user base for their IFC equipment. Discussions were based around how to encourage IFC use in situations, where it is more suited to the research question, along with how to help users put together a successful IFC experimental design. This topic is very dependent on the specific SRL/flow core, those that have heavily used IFC equipment often have extremely good relationships with the principle investigators and have proven the benefits of IFC by publication and repeated successes. It was also noted that canvasing for users, holding seminars, and free trial runs also provides people with information or the ability to test samples at little cost. It was generally agreed that once a successful experiment has been completed, word of mouth and repeated use generally increases.

Since the commercial availability of IFC over the past 13 years, the number of papers published has increased year on year, providing evidence to show the popularity of the technique. Traditional flow cytometry has standard publication guidelines MIFlowCyt (20) to make sure important information is present within manuscripts. Although some aspects of MIFlowCyt can be applied to IFC, especially in the data acquisition side, once into data analysis the difference between the techniques is vast. Due to this, there is no defined way to display IFC masks, features, and images within a manuscript, which can make publication and review difficult. In the workshop, we proposed the publication of a set of guidelines to which IFC data would adhere to in publications. Despite there being some reservations to this there were many attendees who supported at least a set of recommended guidelines for the minimum required information to repeat an IFC experiment and the associated analysis. Such guidelines could help increase the publication quality and make sure IFC manuscripts have all the relevant details required to replicate experiments.

Future Work

The workshop organizers believe that IFC is a powerful technology and will have a place in the field of cytometry for many years to come. The IFC community is growing and this is a good thing; more users mean more exposure for the technology and it applications and challenges. The authors are looking into way to increase the IFC community communication, starting with the google plus forum. The authors are looking into the potential of publishing a guide for SRL/flow cores to increase utilization and maintain users for IFC equipment. This guide might also contain guidelines for best practices for publication of IFC data sets. Increasing the number of IFC events at cytometry conferences would also be a way to help increase the awareness of the technology and its advantages and disadvantages with both researchers and facilities new to the technology.

WS05: Identification of Areas Where Software Tools Can Contribute to the Successful Operation of a Shared Resource Laboratory (SRL)

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Introduction and Aims

WS05 aimed to identify areas where implementation of software tools can help contribute to the successful operation of Shared Resource Laboratories. Our main goals were to identify the areas that SRL members struggled with the most and to report new software tools the community would like to see developed to make facility operations more convenient and efficient. Additionally, we wanted to gather feedback on how existing software solutions currently being used might be further improved. Researching and finding software solutions, which may contribute to a successful operation of an SRL can be a daunting task. The fact that SRL management encompasses different fields in management solutions makes this a great challenge given the immense variety of different options available, varying from very basic tools to highly advanced software packages. The goal of every SRL should be to provide an excellent service to their users and to look for solutions that better suit their specific situation. To achieve this, choices on which software tools to use, need to be made based on multiple variables (budget, ease of use, size of SRL, internal IT services, regulations, etc.).

The target audience of this workshop was SRL managers and SRL members who would like to organize their work in a more structured and efficient way. The expected outcome of the workshop was to assess the interest in creating a list of software solutions for the various tasks in an SRL, possibly hosted at the ISAC website or an ISAC-affiliated discussion forum. This list, generated and curated by the ISAC community, should contain both commercially available offerings, and free or open-source solutions. It could become a very valuable resource for SRLs that are in the inception phase, but also for existing SRLs that are looking for other and better alternatives to the software solutions that they are currently using.

The authors also feel that such resource could be useful for current software developers to take measure of the community's struggles, and that it could identify and prioritize areas to see developed or help to make improvements to the already available software packages.

Methods

In order to gather information beforehand and tailor the workshop discussion, the organizers shared a pre-congress survey, which was distributed via the Purdue flow cytometry list, the ISAC Facebook webpage, and the Google+ cytometry community (https://plus.google.com/communities/107840035426159582772). The first part of the poll focused on existing

software solutions and how they could be improved. The second part had open-ended questions, where the community was asked to identify new areas where software solutions could play a beneficial role. A list with all survey questions can be found as Supplementary Information WS05_SI1 SurveyQuestions. The scope of the survey was to get a broad overview of what SRL managers and members are currently using as software solutions for all the different areas involved in running their core facilities. Different sections were defined: scheduling, laboratory and project management, staff management, data analysis and storage, panel design, communication, and novel areas.

The results of the online survey, with 85 participants (Supplementary Information WS05_SI2 / 3), were analyzed and presented to the audience at the workshop. The slide deck presented at CYTO is available as SI4 (Supplementary Information WS05_SI4 presentation). During the workshop, the audience participated actively via live polling using the Sli.do online polling application (https://www.sli.do/) by answering key questions from the original online survey in order to replicate and confirm if the data generated would be comparable. The live polling results are available as SI5 (Supplementary Information WS05_SI5 slido results). Finally, the workshop audience was given the opportunity to discuss in small focus groups, which software solutions they are using, share their feedback and suggest new ideas for software to develop. A short overview of the discussions by the focus groups was given by the moderators.

Results/Outcome

The survey results of 85 participants were analyzed and an overview of the main outcome, based on the different thematic sections, is summarized below. The raw data of the survey is available as SI2, analyzed data as SI3 and SI4. The participants group was mainly comprised of SRL managers and SRL members. For graphical display and visualization of the results, see Supplementary Information WS05_SI4.

In section 1, the focus was scheduling software participants were asked, which software tool they were using for scheduling equipment and how satisfied they were with the tool. Results showed that 97% of the participants were using scheduling software. Next to the well-known commercial ones (such as Stratocore PPMS and iLab Solutions), a larger part of respondents use in-house developed solutions and free tools such as Google calendar or Booked Scheduler. Following up, attendees were asked for their favorite feature and what in the software could be added or improved. Top features were actual usage tracking, statistics and reporting, responsiveness of software developers, automatic user authentication, email notification, with suggested improvements of mobile app features, billing/invoicing, and search tools. In section 2, questions focused on laboratory management software (used by 62%), and the main applications selected were Google sheets, Microsoft Excel, or in-house developed tools. Key features mentioned were accessibility, adaptability, ease of use, and data tracking. Suggested additions were reagent lot/usage tracking and automation.

Section 3 focused on project management software (used by 32%). The tool mostly referred to was the use of Electronic Lab Notebooks (ELNs), but also commercial applications such as Asana, Trello, and SharePoint were mentioned by the participants. Crucial feature named for the use of these tools was that the project software allows to track development and milestones, besides appending different types of data formats, though improvements could be made in data indexing for quick access to all information. In section 4, the topic was staff management/scheduling (used by 29% of respondents) and top responses were iLab Solutions, Google calendar, and in-house developed solutions. Features like flexibility, linking staff to instrumentation schedules, and tracking the personnel utilization were highly rated.

Section 5 was data management and analysis; here all participants had a system or policies in place to store and backup the produced research data. Cloud solutions were used by some (e.g. Box, Amazon, Google cloud, Nextcloud), where the features highlighted were unlimited storage space, ease of use, and automated scripts. Improvements would be automated cloud storage, automatic data deletion, sharing data with external users, and better IT support. For data analysis, FlowJo was the most used software among respondents, but FCS Express, Cytobank, Kaluza, R, and general acquisition software were also highly ranked. Key features the community would like to see being developed are automatic data clean-up, automatic gating, guided cluster analysis, and 3D visualizations. Regarding panel design, when the participants were asked which tool they prefer, FluoroFinder and the BD Guided Panel Solution tool were the top answers. Suggested improvements were antigen expression level information, tips for users, and spillover spread matrix integration.

The survey's communication section consisted of three parts. The poll results showed the top tools for internal communication were website and intranet pages, email and Slack. Twitter and Facebook were the most popular social media tools, although this category was not used by many. User surveys were mostly done by Surveymonkey or Google forms. The last section of the online survey was the exploration of novel areas, where three open questions were asked: Which novel area do you believe a software tool could make a difference in an SRL? Which existing area/section did we forget? Do you use a tool that you think is valuable for the SRL community and that you want to promote? An overview of the replies to these open questions can be found in Supplementary Information WS05_SI6.

Discussion/Conclusion/Perspectives

The results generated by the survey and the discussions held at the CYTO 2018 meeting show that SRLs around the world are using software solutions to assure a good quality service for their users. Having good software solutions in place can be of great help to adhere to the recently published ISAC SRL Best Practices (52). There is a wide variety of SRLs around the globe, and this is also reflected in the software tools that are being used by the SRLs. Finding out which tool best suits a specific SRL can be a time-consuming

task and extremely demanding, but if done properly, can make a difference in the success of a core facility. A good example of such an exercise is described by Hexley et al. (56). Having, for example, a convenient tool that tracks machine occupancy can justify the investment for the acquisition of an extra one. Similarly, having a proper quality control (QC) tracking software can easily identify problems in early stages and avoid instrument downtime. For certain areas, such as scheduling, it is obvious that almost all SRLs have a system in place. Some software developing companies provide very good solutions for these tasks, which is reflected in the user satisfaction rates. Next to these well-known areas, another goal of the workshop was to identify novel areas where software can help in the SRL and to find gaps in the current software solutions. The survey results delivered very compelling data, which can be of great help for SRL managers who are running or starting an SRL and, of course, can be also useful for software developers to identify new areas or to improve their software packages. During WS05, we were able to compile a list of current and useful software solutions for SRLs. This could be an ideal starting point to develop an online repository where SRLs could find relevant information to make software decisions or share their experiences. The repository could address many different sections (e.g., scheduling solutions, laboratory database management, staff management, project management, quality control systems) and, within each section, proposed software solutions could be discussed (pros and cons, free or commercial, user experiences, etc.). A similar exercise has been performed recently for bioinformatics resources (57). The creation of this repository, its feasibility and format will need to be further discussed and developed at the next CYTO meeting, though based on the feedback from the community there is indeed a great interest in making this repository accessible as a useful resource, both for SRLs and software developers.

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WS10: Best Practices for Development and Implementation of a CyTOF Core

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Introduction and Aims

Mass cytometry promises to be a potential game changing technology that provides unmatched depth of immune system characterization at the single cell level. The method dramatically expands the capability of cell subset identification and phenotypic characterization compared to flow cytometry (FCM). The use of rare-earth metal isotopes conjugated to monoclonal antibodies results in a minimal overlap between channels and enables seamless evaluation of multiple parameters simultaneously on individual cells. The difficulties in this platform typically concern 1. ease-of-use, 2. high cost, 3. creation of large panels, and 4. data analysis and visualization (Supplementary Information WS10_SI1), all of which can be limiting factors or roadblocks to the widespread implementation and use of this technology compared to flow cytometry.

The workshop was held to aid flow cytometry labs who are seeking to establish mass cytometry capability in their lab, but may have struggled with implementation due to any number of unforeseen challenges. We intended to discuss these challenges and adapted solutions from experts in the field who both operate flow and mass cytometry cores.

The CYTO Workshop 10 was held on 30th April 2018. Approximately 90 users attended the meeting. Attendees were mainly scientists from academia with a few from Biopharma and clinical labs. Most participants completed a pre-workshop survey (Supplementary Information WS10_SI2). The objective of the survey was to reveal consensus answers to some frequently asked questions and provide an information resource for laboratories looking to integrate a CyTOF capability or service.

Well recognized leaders from both mass and flow cytometry helped guide the discussion and challenged the presenters with thought provoking statements and questions. The discussion integrated multiple perspectives, which enabled the identification of solutions that could provide sufficient assurance for the intended usage of mass cytometry.

Methods

The format of the workshop was comprised of four 5–7 min presentations by different facilitators in an open dialogue allowing for real time attendee participation (Supplementary Information WS10_SI1). The four key focus areas were comprised of:

1. Ease of use
2. Cost savings and reagent management
3. Utilization of automation and robotics
4. Data analysis and visualization methods

We prepared a pre-conference survey questionnaire (Supplementary Information WS10_SI2) to address each of the four key areas to facilitate discussion. We created the survey link (https://www.surveymonkey.com/r/2G7Q7SG and posted to the Purdue Cytometry email list (58), Cytoforum email list (59), CYTO 2018 workshop website, and local user group lists before the workshop to collect information from cores currently utilizing or struggling with the technology. We received 48 responses from around the world.

The facilitators took notes on the discussion and attendee feedback questions and statements, which were summarized for this report. Survey results were discussed after each presentation (each discussing a different topic) followed by an open-mic session. The workshop, which was originally planned for 90 min as per the guidelines from ISAC workshop committee, ran long by 20 min due to active and

frequent audience participation. The conclusions proposed in this report are due to well-drafted presentations (Supplementary Information WS10_SI1), the inclusion of pre-conference survey (Supplementary Information WS10_SI2), the discussion of survey results (Supplementary Information WS10_SI3), and live discussion, which was combined to generate a single report and analyzed for common needs and gaps in our current knowledge.

Results

Ease of use. Sixty five percent of the respondents indicated that CyTOF assays are frequently reliable and repeatable. However, from the discussion, many labs were unclear on proper methods for the titration of antibodies, as well as instrument sensitivity. The major challenges for CyTOF implementation are panel development and optimization, antibody resolution, standardization, and data analysis. Fifty four percent of the labs took less than a year and 38% took around 1–2 years to implement the technology. It was concluded that at least three full time employees are required to run a mass cytometry core. Seventy percent of the responders were satisfied with sensitivity relative to FCM, although a few commenters expressed that sensitivity can vary between instruments and between tuning procedures, and that the same standards that are used in flow cytometry are not present in mass cytometry.

Cost savings. Sixty six percent of respondents agreed that CyTOF is expensive with respect to reagents and instrumentation compared to running a flow experiment; 75% agreed that technology is limited due to the lack of multiple vendor supplied reagents, with one commenter expressing that the supply of instrument parts was an issue as well as the provision of reagents. Most users agreed the fact there is only one commercial vendor limits the support needed for the technology.

Automation. The approach of utilizing robotics or automation is to reduce variability. Many labs are unaware of, or have never attempted, automation except for a select few (such as the laboratory of Garry Nolan), and some pharma companies were developing in this area. Instrument to instrument variability and reliability could be evaluated with controls for better instrument performance characterization, which are greatly needed.

Data analysis. Data analysis and visualization methods are questionable given variability in results generated by clustering algorithms. While Cytobank is the premier mass cytometry analysis platform, more powerful and adaptable tools are required.

Many of the existing clustering and dimensionality reduction tools can be run in R packages, such as the "cytofkit" package (60) (https://github.com/JinmiaoChenlab/cytofkit). While tools like SPADE, viSNE, and Citrus are sufficient for basic representation of data, the continued progression of machine learning offers the potential for new

approaches to be developed that will help advance the analysis of CyTOF data.

Discussion/Conclusion/Perspectives

Ease-of-use. While the majority of the respondents of the survey indicated that CyTOF assays are reliable, our discussion on this section was largely focused on titration of antibodies (61) and visualization of background. It was recommended through discussion that the approach with the greatest confidence was optimizing resolution from background. In this scenario, the maximum amount of antibody is used that does not result in an unnecessary increase in background binding, resulting in the maximum resolution of signal from noise. There should be no difference in methods of titration used in mass cytometry or flow cytometry, as both use reporters conjugated to monoclonal antibodies. It was stated that most mass detection channels have a background between 0 and 1 and that spillover could be the main source of increased background. Additionally, there were comments that sensitivity and specificity could be evaluated through a comparison of two identical clones with different fluorophores/isotopes stained on the same cells. A nondiagonal plot indicates a pair of nonreproducible reagents. Due to the complex nature of the technology, dimensionality of data, and need for customization, it was unanimously concluded that employment of at least 3 full time staff is optimal for running a CyTOF core. The duties include: employee 1. Reagent management, Conjugations, Staining, Titrations; employee 2. Instrument operation, maintenance, sample acquisition, training of new users; employee 3. Data Analysis could be outsourced from bio-informatics depending on the resources. In some facilities, this role covers mass and flow cytometry data analysis.

Survey respondents also indicated the challenges of infrastructure that delayed the installation of a CyTOF in their facilities. They include building adaptations, space and HVAC (heating, ventilation and air conditioning) systems inclusions, venting and exhaust-related issues.

Cost savings. Optimization efforts to build a CyTOF panel, low sampling efficiency, long run time, inclusion of EQ4 beads, inclusion of reagents to exclude dead cells, and tweaking commercially available pre-configured CyTOF kits are the various factors that make CyTOF experiments more expensive than flow experiments. Validating CyTOF reagents under relevant conditions (i.e., permeabilization or stimulation) that may affect the level of staining is an important aspect of proper experimentation, resulting not only in improved assays, but also in cost savings. Control antibodies and gating antibodies may be needed during titration since large panels often measure immune-modulated antigens. Attendees shared ideas that would benefit the shared resource laboratories (SRLs) who manage reagents, such as the need for resources for commercial clone-isotope validation, resolution, and titration resources similar to those created for flow at the NIH. Given their central involvement with the technology, SRLs are valuable collaborators for pilot studies where in-house reagent conjugation, titration, sensitivity testing, and

controls are necessary for most panel optimization efforts. Finally, billing approaches (fee-for-service labs) for investigators could be offset with collaborative proposals where core recognition in the form of authorships may help contain costs and enable improved lab/PI relationships.

Automation. Integration of robotics and automation platforms for the preparation of antibody cocktails, reagent management, and cell processing is important to control contamination, improve data quality, improve reproducibility, and reduce variability (62).

Sub-microliter reagent volume titrations without reagent dilution, combined with reduced cell volumes (assay miniaturization) contributes to cost savings. Creating CyTOF panels to generate high dimensional data is fast and accurate with fluid handling systems.

Although many attendees found the prospect of automation exciting, for many the cost was a prohibitive factor. However, in many cases, these modular systems are no more expensive than high-end cytometers. It was suggested that laboratories that are utilizing robotic instrumentation could help support the community by posting and sharing data on antibody staining as discussed above.

Data analysis. Despite a decade of data generation, a major bottleneck in the implementation of mass cytometry is the management and analysis of the resulting high-dimensional data sets. While many computational approaches exist, a key difficulty is a lack of understanding of the proper use of these tools, in addition to a lack of understanding of the raw data being used for analysis. This imbalance inevitably leads to a reliance on the output from computational tools, whether erroneous or not. As such, education here is a key to the generation of reliable data. Moreover, while many groups may employ, or have access to, separate biological researchers, technologists, and data analysts; sufficient overlap of skills in these areas in each individual are necessary to ensure a harmonious workflow of data generation and analysis. Simply, without due care, attention, and education, poor choices in analysis risks the generation of large volumes of poor-quality data.

The tools used in the analysis of high-dimensional data sets are constantly evolving and improving. While the most common solution is the Cytobank platform, many computational analysis tools are available as open-source packages in programming languages such as "R" (63). These tools allow for modification and experimentation of existing analysis approaches but require a knowledge in informatics and the use of R. On the other hand, GUI-based tools like Cytobank allow a wider base of researchers to harness powerful analytic tools. In either case, multiple benchmarking (64,65) and comparison studies aid us in the assessment of the relative strengths and weaknesses of different tools. Where possible, the same data set should be analyzed by a number of approaches, as deficits in one approach may be supported by other approaches.

Another critical element in ensuring the generation and propagation of high-quality data is transparency in publishing. It was suggested that data sets used in published materials should be uploaded to an online repository, such as flow repository. This allows both reviewers and readers to assess the analytical approach, and the conclusions drawn from it.

Overall, while mass cytometry provides large amounts of high-dimensional single-cell data, continued discussion and development of best practice guidelines within our society is necessary to ensure the generation of high-quality mass cytometry data in the future.

CHAPTER 2: SHARED RESOURCE LABORATORY (SRL) BEST PRACTICES CONCLUSION

Through these four workshops those who work in an SRL environment will benefit from the active discussions on the challenges, as well as the proposed ideas to address them. The results of these workshops, along with their supplemental information and resources, provide a wealth of knowledge and innovation from which the readership can draw upon for consideration and application in their own SRL setting.

Successful SRL has top notch instruments, operators that are knowledgeable enough not only to perform measurements but to analyze obtained data, train other scientists on measurements and analysis and attract new customers, so that SRL can afford new instruments. Even though it may sound simple, execution of all the steps in this cycle is not that trivial. Experiments involving new technologies are expensive and in many cases, it is difficult to convince the users to employ them in their studies. Organizing free trainings, public presentations or direct discussions with end users can positively impact the occupancy rate of the instruments. To convince the community to use new technology and organize training, SRL staff must balance their own professional development between science and technology. However, this takes time, and funding would be beneficial to SRL and attract potential new staff members interested in two hats jobs. To facilitate flow of information and assure data reproducibility, knowledge database and repository should be regularly updated and enriched with relevant new technological features and associated procedures.

Chapter 3: Quality Assurance and Reproducibility

Flow cytometry is a powerful technique used in basic biological research to critical decision-making in a clinical environment. Four workshops were held to evaluate the current status of quantitative measurements in flow cytometry. Much has been achieved in standardization and harmonization of flow cytometer applications in multicenter clinical studies (WS03). However, there is an urgent need to accelerate the development of quality material and documentary standards (WS09). Preserved cell standards need improved shelf life, preservation of labile markers, and increased resolution of dim populations (WS13). Estimating sample stability and preanalysis processing is problematic without appropriate standards. Validation of assays is still largely on an assay by assay and investigator by investigator basis. High-quality standards would enhance the veracity of the assay validation process. Standards for flow cytometer setup, calibration, and compensation are needed to assure measurement confidence and comparability of results obtained from different instrument platforms, laboratories, and times (WS11). Data analysis procedures, such as gate settings, need constant attention and upgrading.

WS03: Dr. Reproducibility or: How I Learned to Stop Worrying and Love Validation

Steven Eck, Christopher J. Groves, Jennifer J. Stewart, Yongliang Sun, Michael Hedrick, Cherie Green.

Introduction

Given the risks that irreproducible results pose to the advancement of science, every scientist should be looking for ways to ensure their work is replicable. Analytic validation is a process that begins with good instrument control, uses good assay development practices, and culminates with demonstration-through-experiments that the assay adequately makes its measurements to support the scientific conclusions (i.e., that the assay is fit-for-purpose). Validation can be an iterative process in which the extent of understanding of the assays performance can increase along with the importance of the results in a risk-based fashion. As such there is no good reason not to apply principles of validation for nearly any assay that needs to be able to support reproducible science.

Aims

The goal of this workshop was to provide a brief overview of common validation principles and to present and discuss real-life examples that illustrate common validation experiments. The intent was to demystify validation and illustrate it as a thoughtful, science driven process in which the amount and stringency of the validation experiments performed can reasonably be risk-associated with the intended use of the assay.

Conclusions

While the first-third of the session was largely tutorial around analytic validation and instrument control, the remainder of the session focused on validation examples. Among the more interesting conclusions were that the attempt to achieve precision CVs of 20–35% as found in white paper guidance's was nearly universal as was acknowledgment that this could not always be achieved. Similarly, it was acknowledged that precision standards were rarely more stringent than those above. There was also general agreement that the extent of validation should be commensurate with the importance that the conclusions from the assay be correct, but refining that concept to practice is still largely on an assay by assay and investigator by investigator basis and as such would benefit from additional guidance.

Perspective

Validation uses predetermined experiments to verify the performance of an assay. While it's broadly used for drug development assays, the practice is not well adopted in basic research. The workshop served to illustrate how aspects of validation can be simply accomplished, highlighting consensus and still evolving aspects of validation in flow cytometry.

Introduction

The most basic requirement of research is that it should be reproducible. Validation science focuses on identifying experiments that can most powerfully inform us as to the reproducibility of the assays we use to generate our data. In settings where validation is required by regulation, the formality of it can seem daunting. In practice, the most commonly used validation experiments are straightforward and can be accomplished with a reasonably modest effort on the part of the investigator. Fit-for-purpose is a guiding principle of analytic validation that dictates the assay performance must be adequate to assess changes in the measured analyte. However, by its nature fit-for-purpose assay validation is interpretive. The goal of the workshop was to introduce common validation practices and considerations through real-life examples to generate discussion and, where possible, consensus around issues associated with validation-related decisions.

Methods

The workshop was presented by panelist experts in flow cytometry analytic validation drawn from the Flow Cytometry Action Community of the American Association of Pharmaceutical Sciences (AAPS). The first section had two individual speakers providing a brief overview of analytic assay validation principles and instrument performance control. The second section had three speakers presenting case studies with questions and discussions following each section and case study. Questions solicited from the audience (of roughly 70 people) formed the bulk of the discussion.

Results/Outcomes

The session dealt with basic tutorial concepts in validation moving into animated discussion around parameter

selection for fit-for-purpose assay validation through case study examples. In the context of instrument performance control, questions arose on the use of the different cell or bead-based technologies and frequency of use (daily, with each assay, etc.) and it was concluded that this could differ for different situations depending on assays in use.

In the case studies, application of the concept of fit-for-purpose validation was a repeated topic with discussion around the selection of passing CV criteria. Similarly, a presentation of an assay used to address futility (secondary endpoint) in a clinical study brought up questions and discussion on the difference in validation between a secondary endpoint versus an exploratory-use assay. Finally, the presentation of a receptor occupancy assay prompted discussion of which experiments fall into the category of assay development and which are the part of the validation process. While agreement in a broad sense was achieved, some differences in opinion were present. As a result, from the conference, the audience and panelists obtained a sense of the validation process as currently applied in real-world settings on real examples. The discussion and questions provided a general perspective on what kinds of topics have broad agreement and which remain interpretive.

Discussion and Conclusions

The overview of the validation process provided at the beginning of the session including the brief summaries of the different validation parameters was in keeping with that of flow cytometry-specific protocol collections and white papers (66–68). The concepts of "fit-for-purpose" assays and the iterative approach for validation were presented with the scope and the scale of the validation required increasing relative to the importance of the data the assay generates (limited for basic research to most complete for regulatory submission for marketing approval (69,70)). This concept is illustrated in Figure 3. An example of a two analyst two investigator study was presented with an emphasis on how this could be accomplished within 32 tests basically comprising as little as one testing day for the analysts (Supplementary Information WS03_ Supplemental data slide).

The overview of cytometer performance maintenance presented processes to ensure stable instrument performance (28,52,71,72). There was general agreement around the importance of educating the user base as well as on the use of beads and daily QC's and defined cocktails. The latter were in-line with recommendation found in recent publications (52,71,72) and a recent NIST-FDA flow cytometry workshop on building measurement assurance (73). Discussion was held around the frequency of use of reference material (each day, each run, etc.) reaching the conclusion that frequency and use of the different kinds of materials available differed according to application. There was also general agreement on the utility of robots for cocktailing to reduce time and error relative to human generated cocktails though this topic was not expounded upon further.

The session then went into a series of analytic validation case studies including a Natural Killer (NK) cell functional assay, a receptor occupancy assay, a cell depletion assay, and a T cell activation assay. The first set of examples highlighted the importance and usefulness of applying validation-specific experiments during assay development and optimization steps such that the validation study becomes the demonstration of what is largely already known about the assay performance. Questions came from the audience about the selection of the pass/fail precision criteria, and whether it was just adopted from literature proposed values or was done in consideration of the use of the assay. The answer in the discussion that followed was somewhat complex, with the speaker iterating that in this case because the assay could easily achieve precision in-line with stock literature CV values (e.g. 25–35%, (66–68), before panel discussion progressed to the idea that for assays unable to achieve such precision a "fit-for-purpose" approach might allow higher CV's to be permissible so long as it was taken into account in declaring the meaningful interval between measures. An example of monitoring changes in Ki67 expression within cell sub-populations where only a CV of 40–50% might be achieved was discussed. It was argued that such a CV might be inappropriate to look at relatively small changes but might be perfectly capable of determining if multiple fold changes were occurring. This point was expanded further with the explanation that the increasing total analytic error essentially increases the breadth of distribution around true mean and median and in studies where the intent is comparison between groups rather than individuals. In these cases, a power analysis type approach can be applied such that the difference between distributions should be provable as statistically significant. Thus, it should be possible to validate such values following the rationale of "fit-for-purpose". While this view is not always presented in some of the current white papers on flow cytometry assay validation, it is in keeping with some of the prominent publications on biomarker assessments (69,70).

In drafting this summary, the panel aims to emphasize that it encourages the use of promulgated 25–35% maximal CV's listed for quantitative assays within many of the white papers. These CV's largely evolved in the examination of pharmacokinetic assessments, reflecting the differences between mass spectrometry approaches used in small molecule versus the ligand binding assays used in large molecule drug development and have an established history with regulatory agencies. Justification that the assay precision is adequately "fit-for-purpose" should be provided if precision CV criteria are substantially higher than these values are used, and such assays should likely be regarded as semi-quantitative. Selection of precision CV's for biomarker assays in general remains a contentious issue, which has arguably not decreased following the FDA's finalization of its PK and biomarker guidance (74). Of note, in this panel's estimation, most flow cytometry assays, with the possible exception of assessment of target antigen expression quantitation, are likely out of scope for that guidance.

The remaining topics involved less discussion and were generally well agreed upon. They included discussion of

The iterative approach to validation:
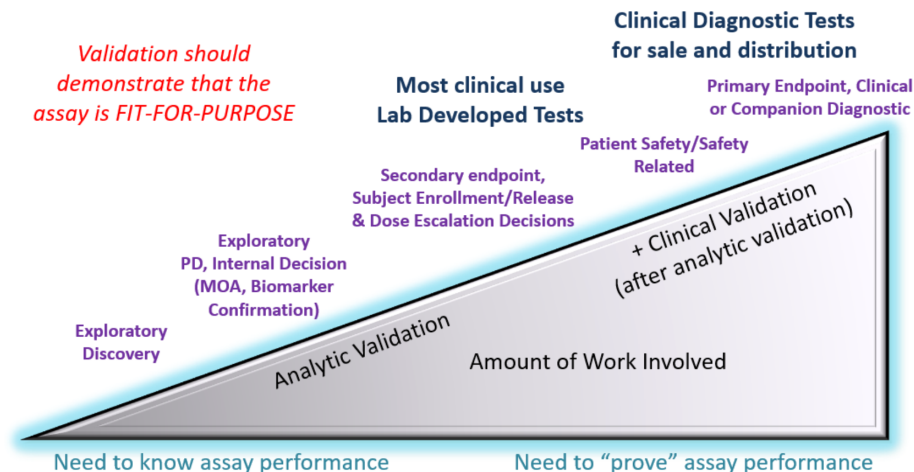### *Reasonable relative to the goal at the time*

Figure 3. Illustration of the iterative approach to validation. [Color figure can be viewed at wileyonlinelibrary.com]

whether precision acceptance criteria are ever made more restrictive because the assay in development demonstrates better than literature recommended values. In this case, consensus was that while pass/fail criteria might be loosened following fit-for-purpose considerations, they are very rarely tightened (if ever) and there would probably need to be a compelling reason to do so as it would involve risk for future assay failure. For example, it is possible that performance under maintenance conditions (such as when incorporating new lots of antibodies) might deteriorate and become at risk of not achieving unnecessarily restrictive CV's relative to what was needed for the assay's utility.

Another interesting discussion followed presentation of a receptor occupancy assay development around the utility and requirements for looking across a receptor occupancy curve (different drug levels) for stability. In the example provided the in vitro assessments highlighted 72-h stability risks associated from two of the populations (CD4 and CD8 T cells) that were not apparent in the B cell population. In the ensuing discussion, it was accepted that the data was useful but not always part of a validation package given the considerable presumptions made; namely that the post collection stability associated with an equilibrium established in vitro will be similar to that of an equilibrium that has established in vivo. For example, if the drug has activation or depletion capability, there may be considerable selection in vivo that could impact postcollection in vitro stability. Numerous topics around receptor occupancy considerations are found within the *Cytometry Part B* special edition on receptor occupancy (75), and this discussion adds to those.

The session ended on presentation of two assays, a B cell depletion assay that was a secondary endpoint (futility decision enabling) on a clinical trial and an exploratory T cell activation assay. The juxtaposition prompted discussion on the validation differences between exploratory and secondary endpoint assays. Agreement was clear that secondary endpoint assays often

differed in both scale (number of samples used to validate each parameter) and scope (number of validation parameters assessed). Precisely how they differ was presented as something determined uniquely for each situation. In the example, it was pointed out that for the more exploratory assay Lower Limit of Quantification was not considered, while it was a critical consideration for the secondary endpoint assay. In discussion, there was broad agreement that as the result of an assay becomes more important, the proof that the assay is well controlled and capable of making the meaningful measures becomes greater (both in the scope of parameters validated and the numbers of samples used). This fits well with the iterative approach to validation; however, there is still little firm guidance on how specifically to apply this concept in selecting specific validation experiments to perform relative to an assays risk categorization (as in the above case where it would play a more prominent role in supporting the drugs approval). Hopefully some of this ambiguity will soon be addressed as there is a Clinical Laboratories Standards Institute (CLSI) endeavor to produce a guideline for analytic flow cytometry assay validation, which hopefully will be open for public viewing and comment in spring of next year (Guideline H62: Validation of Assays Performed by Flow Cytometry).

In summary, the session provided a well-rounded overview of current topics in validation and obtained consensus opinion on the points described above.

WS09: Control Cells or Not

Paul Wallace, Jonni S. Moore, Derek Jones, Virginia Litwin, Lili Wang, Yanli Liu.

Introduction and Aims

Biological controls can be used as positive, negative, or process controls; they can also be used for instrument set-up or standardization; reagent quality controls; panel characterization;

and for multiinstrument and multicenter longitudinal studies. Both normal and abnormal controls are desired to prove that assays and reagents are working appropriately. However, effective and stable control cells are difficult to secure for cell-based assays. The challenges of current preserved cells include short shelf life, deteriorated preservation of labile markers, and decreased resolution of dim populations. This workshop was proposed as an in-depth discussion to the challenges that we are facing as a community.

There were three primary objectives for the workshop: 1. an educational session on control cells; 2. a forum to discuss whether the inclusion of biological controls in an assay or experiment is necessary and a best practice; and 3. an opportunity to identify and initiate potential collaborations to form consensus on controls and define future strategy.

The audience of this workshop included researchers, laboratory scientists, clinicians, and vendors in the cytometry community and adjacent fields, such as imaging and genomics. This workshop was designed to define the pathway to overcome the challenge of the lack of readily available biological control materials for the following tasks:

- Establishing baselines to routinely monitor the quality of reagents and assays;
- Standardizing longitudinal studies for accurate and consistent results in research or clinical settings;
- Standardizing parallel studies across instruments, platforms, and centers;
- Implementing a standardized workflow to reduce laboratory cost and waste, and to increase the credibility of the laboratory; and
- Automating and standardizing data analysis to usher in the new era of computing and quantitative cytometry.

The expected outcome of this workshop was to find better solutions to the key challenges of biological controls, establish consensus on control cells, and improve the quality of research and clinical studies in academia and industry.

Methods

Prior to the session, an electronic poll consisting of 13 questions was conducted through the CYTO2018 workshop website and CYTO2018 mobile applications to gather preliminary input from ISAC members. We sought to understand the currently used controls, frequency of usage, regulatory requirements, the biggest challenges in their use, and the top three performance features required by end users.

Then four technical presentations covered the significance of using control cells and the major existing biological controls from different vendors. Lili Wang showcased data on using cell reference materials for measurement assurance in flow cytometry and cited the usefulness of the characterized human peripheral blood mononuclear cells (PBMC) preparations as controls for sample processing, antibody reagent quality, and panel optimization. Paul K. Wallace presented a study using fresh whole blood and two types of preserved cells as procedural controls to confirm the day-to-day reproducibility of the assay, the reagents, mAbs, buffers, technique,

and instrumentation. Derek Jones and Jonni S. Moore demonstrated the potential of using control cells as a method for cross-site standardization to enable translational research. A comparison was made of commonly available control cells on parameters such as light scatter, markers expressed, stability, and cost. Representative staining profiles were also presented. Finally, Virginia Litwin presented three case studies of implementing controls in her studies.

The last part of the workshop consisted of an interactive discussion with panelists as well as a short online survey covering several critical topics such as: current controls in use, performing quality control of instruments, reagents, or assays, the challenges and the sources of the control materials, and the lab requirement of regulatory status of control cells in order to adopt them in research and clinical studies.

Results

Fourteen responses to the pre-workshop survey were submitted prior to the workshop session. About 40 attendees participated in the live polling during the workshop.

When asked what type of controls were used in their studies, users reported a wide range of control materials: beads, stained/unstained/unstimulated cells, lyophilized Veri-Cell™ PBMC, and frozen cells. The frequency of using controls for instruments varied between experiments, daily to weekly, depending on the goals and scope of the studies. All participants used beads to perform quality control of instruments. The frequency of using controls for reagents appeared less frequent. Some users reported "hardly ever" or "sometimes" regarding the frequency of usage, while others reported frequencies from "daily" to "every time lot number of the antibody changes", or "just when we see potential issues and when setting up a new experiment". Materials implemented for reagent quality control included fresh blood, beads, preserved control cells, and others.

Which assays are required to use controls? Some attendees responded that *every* assay needs controls and others replied that controls are needed especially for lab-developed tests (LDTs), apoptosis assays, microvesicle assays, and immunophenotyping. Examples of critical markers were death markers, drug target markers, tandems and low-expression markers, toll-like receptor markers, neuronal cell markers, and immune memory markers. The top required features for control cells were consistency, reliability, ease-of-use, flexibility, stability, and affordability.

During the lecture session, Lili Wang discussed the usefulness of characterized human PBMC preparations. Paul K. Wallace presented data, including 10-day Levey-Jennings plots, on tests conducted with three control preparations. He pointed out that bulk dried cell preparations performed better than fresh cells, and kappa and lambda light chain were resolved on fresh blood and BD Horizon® Dri Leukocytes. Based on his evaluation of several commonly available control cells, Derek Jones recommended the use of control materials for standardization of different instruments across multiple sites and over time. He also noted that BD Horizon® Dri Leukocytes appear to outperform other control materials based on

his evaluation of markers expressed, stability, and affordability. To conclude the presentation session, Virginia Litwin demonstrated that control cells were found to be important reference material to monitor variation between daily runs, assays, instruments, analysts, and to identify reagent issues.

Discussion

Controls are used to establish a baseline to compare results against introduced variables in cytometry. Control cells can be used as positive and procedural controls for flow cytometric applications such as phenotyping of leukemia and lymphoma, immune monitoring and drug discovery. Scientists have been exploring cell preservation techniques to achieve both consistency and performance for a few decades (68,76,77,83). This workshop covered all current products as well as laboratory-developed approaches.

The following key technical issues were discussed during the panel session:

- Assay values and expected ranges of percentage positive cells for specified markers on all instrument platforms are desired. This is important for the lot-to-lot transfer of the QC tests as well to be able to use different instrument platforms. However, current expected ranges provided by the supplier are often too wide to provide practical details for specific markers on specific instruments regarding a particular lot. This remains a challenge for the field due to the complexity of both markers and instrumentation.
- Large lots of control materials are desired in order to standardize results across instruments and laboratories; an alternative was suggested of comparing the lot in use with the incoming lot when a large lot of control materials is not readily available.

Control cells are important yet remain challenging for the field of flow cytometry. Forming a consortium, for example, Flow Cytometry Quantitation Consortium, to visit the topic annually will enable advancement in the field and establish collaborative efforts to share best practices. The Control Cell Workshop Committee is committed to advancing the field forward to provide better quality of research and clinical results for years to come.

Acknowledgements

WS13: Building Measurement Assurance in Flow Cytometry

Lili Wang, Stephen Perfetto, Robert Hoffman, John Elliott, Sheng Lin-Gibson, Steven Bauer, Heba Degheidy, Judith Arcidiacono, Virginia Litwin.

Introduction and Aims

This report summarizes key findings, including the need for high quality reagents, reference standards or materials, and documentary standards.[1] Advances of cell-based therapeutics have increased the need for high quality, robust, and validated measurements for cell characterization. Flow cytometry has emerged as an important platform due to its ability to rapidly characterize heterogeneous cell populations and subpopulations. For example, flow cytometry was critical for establishing identity, purity, and potency for CAR-T cell manufacturing (78); and associated data supported the approval of Biological License Applications (BLA)[2] by the U.S. Food and Drug Administration (FDA) and the approval by the European Medicines Agency (EMA). In addition, multiparameter cytometric measurements are routinely carried out in vaccine and cancer research, clinical diagnosis, and immunotherapies. However, challenges remain with respect to measurement confidence and comparability of results, hindering critical decision-making based on flow cytometry data.

As a part of the joint efforts to develop standards to support innovation and translation of regenerative medicine products, NIST and FDA hosted a workshop titled "Building Measurement Assurance in Flow Cytometry" in October 2017. Approximately 70 experts representing industry, academia, and government agencies attended the workshop. Most participants completed a pre-workshop survey (Supplementary Information WS13_SI1). The survey indicated that the most industrial and academic participants used flow cytometry as a release assay and/or for monitoring of cell manufacturing process (Supplementary Information WS13_SI2). The workshop agenda and presentations are available online (73).

WS13 was held in April 2018 to continue dialogue with respect to broader cytometer users for identifying application challenges and potential solutions. Nearly 100 participants joined the workshop, where the majority use flow cytometry for basic medical research, contract research organizations (CROs) under the category of 'other,' or clinical disease diagnosis (Supplementary Information WS13_SI2).

The different user perspectives from the two workshops enable us to identify common measurement challenges and actionable solutions for obtaining sufficient assurance for the intended flow cytometric measurement.

Methods

For the joint NIST-FDA workshop (73), a pre-workshop survey (Supplementary Information WS13_SI1) was used to gauge participants' experiences and identify common challenges. In addition to introductory content, the one-day

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[1] A "documentary standard" is a classification, guide, practice, specification, terminology standard, or test method developed and established by knowledgeable people according to agreed principles of consensus, such as those of ASTM (American Society for Testing and Materials) International.

[2] A biologics license application (BLA) is defined by the U.S. Food and Drug Administration (FDA) as follows: The biologics license application is a request for permission to introduce, or deliver for introduction, a biologic product into interstate commerce (21 CFR 601.2). The BLA is regulated under 21 CFR 600–680.

workshop covered three general topics: 1. standardization strategies, 2. biological and nonbiological reference materials, and 3. best practices and use cases from two CROs and the clinical laboratory at National Institute of Health (NIH). The first two topics were followed by discussions on calibration and reference materials. The last session was followed by guided discussion on strategies for moving forward in the areas of quantitation, gating, and best practices.

For WS13, the content of the NIST-FDA workshop, including the survey and presentations, was provided as the pre-workshop information on the CYTO 2018 webpage. The workshop consisted of four 10-min presentations that covered measurement assurance concepts, instrument standardization and detector operating voltage optimization, cell-based reference controls, and information regarding a CLSI effort to develop a guidance document for flow cytometry validation (Supplementary Information WS13_SI3). The workshop presentations were followed by live polling of selected survey questions and discussions.

Survey responses from both workshops as well as live polling results from the WS13 have been combined to generate a single report identifying common needs and gaps (Supplementary Information WS13_SI2).

Results/Outcome

The most widely reported survey respondents used multiparameter flow cytometry assays (either 6–12 colors or > 12 colors) to investigate blood samples or cell lines (Graph #3 of Supplementary Information WS13_SI2). The most common objectives were to quantify specific cell subsets and characterize the mean fluorescence intensity (MFI) of antigens they express (Fig. 4A). A generalized flow cytometry measurement process for an individual laboratory consists of roughly six major steps: sample collection, cell processing and staining, cytometer QC, calibration and standardization, compensation, quantitative measurement, and data analysis and reporting as shown in the first column of Table 4. Some survey questions were intended to highlight participants' assurance controls for each process step. Most respondents used a combination of beads and cells to perform cytometer compensation as well as manual gating for data analysis (Graph #8 and #9 of Supplementary Information WS13_SI2).

The most widely reported responses indicated that cytometer standardization is of a high priority to their organizations due to the use of different cytometer platforms or at different sites (Graph #12 of Supplementary Information WS13_SI2). In spite of the fact that close to half of the respondents previously participated in interlaboratory studies comparing flow cytometry results, most are not satisfied with or are uncertain about compatibility across instrument platforms or sites (Graph #13 of Supplementary Information WS13_SI2). The three biggest obstacles for obtaining high confidence assay results are 1. a lack of high quality reference materials, 2. lack of confidence in the procedures from standardization/inter-laboratory studies, and 3. uncertainty associated with specimen quality and/or pre-analytical processes (Fig. 4B). A key workshop finding is the

need for high quality reference standards as shown in Figure 4C. There remain significant needs for common control materials, documentary standards, well-defined measurement procedures, and proficiency training studies as well (Fig. 4C).

Discussion/Conclusion/Perspectives

Measurement assurance requires a systematic approach that informs the confidence in a measurement, and hence the comparability of results. Reference materials, process controls, experimental design, quality by design (QbD), assay validation, and interlaboratory comparisons are examples of measurement assurance strategies needed for achieving traceability and uncertainty qualification of the results (79,80).

The primary goal for utilizing flow cytometry (Fig. 4A) is to measure specific cell subsets and antigen expression; yet quantification via flow cytometry remains a challenge. Significant sources of variability can be introduced at each of the six major steps in the flow cytometry measurement process as well as entitle assay performance/standardization across different locations (Table 4). Measurement assurance strategies, particularly the use of reference methods and materials, could effectively minimize these sources of variability (Supplementary Information WS13_SI4). Workshop experts agreed that appropriate use of these and other strategies can improve their confidence in measurements made by Flow Cytometry (Fig. 4B).

As multiparameter flow cytometry assays are increasingly used to simultaneously characterize and quantitate multiple cell subsets and their antigen expression, a primary requirement is that all detector channels have sufficient sensitivity and resolution to identify and measure the full range of cell surface antigen expression levels, especially the antigens with low abundance. In addition, fluorescence channels should be operated within a linear range (nominally within 2%) to ensure correct spectral compensation. To address these concerns, well established practices and methods are recommended. A set of multi-intensity beads (unstained and stained, including dimly stained fluorescent beads) with defined fluorescent intensity units is deemed adequate to characterize cytometer performance with respect to linearity, dynamic range, electronic noise, sensitivity (Q), and background (B) (81,82). The (CV) for the brightest microsphere population can be used to assess the laser alignment to the sample core stream. The fluorescence intensity unit can be expressed or normalized via the numbers of equivalent reference fluorophores (ERF) assigned by NIST (83). The use of a pulsed LED source can provide consistent evaluations of Q and B without added variability inherent to beads and optical alignment (82). Q and B provide the flow cytometer's ability to resolve dim fluorescent populations from negative populations (84). In this overall scheme, assigning ERF values traceable to NIST SRM 1934 (85) is essential for the standardization of cytometer performance characteristics and enables comparability in multi-instrument and/or multisite longitudinal studies, which should address attendee concerns for adequate assurances (86). Considering that users largely rely
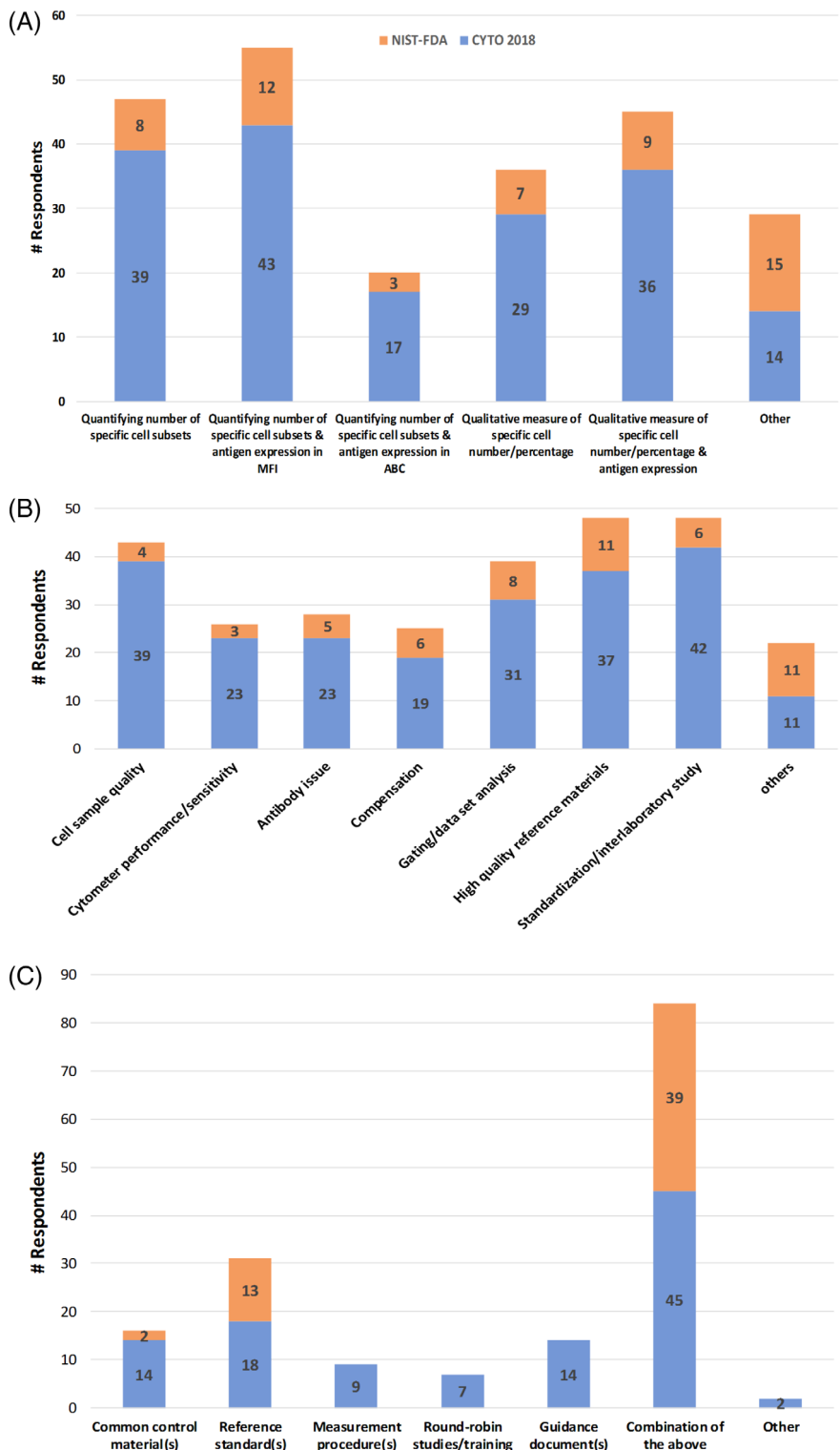
Figure 4. Illustration of the survey results from CYTO 2018 WS13 (Blue) and NIST-FDA Workshop (Orange) on (A) objectives of flow cytometry assays, (B) the biggest obstacle in flow cytometry assays performed, and (C) tools that would help most to achieve measurement assurance.

on built-in software for instrument QC and sensitivity characterization, and there are very few cytometer QC beads with fluorescence intensity values assigned traceable to

available NIST standards (Graph #6 and #7 of Supplementary Information WS13_SI2), significant improvements can be made through a joint effort from instrument

Table 4. Flow cytometry measurement process, sources of variability, reference methods, control materials, and procedures

MEASUREMENT PROCESS	SOURCES OF VARIABILITY	REFERENCE METHODS, MATERIALS, AND PROCEDURES
Sample collection	• Fixed versus fresh samples • Anticoagulant • Cell count and viability • Cell debris	• Counting bead reference • Cell (live/dead) reference control material(s)
Cell processing and staining	• Antibody quality: fluorophore labeling quality, binding affinity, and titer • Cell debris	• Method(s) for evaluating antibody quality • Cell reference material(s)
Cytometer QC, calibration and, standardization	• Linearity, sensitivity, and resolution • Instrument threshold and voltage setting • Volumetric cytometers: volume calibration	• Bead reference materials • Beads or beads/LED methods
Compensation	• Linearity range • Choice of labeling fluorophores/panel design	• Compensation beads • Cell reference material(s)
Quantitative measurement	• Tube-to-tube variability of counting beads • Cell reference material(s) with known cell concentration and/or antigen expression • Assay format (single tube or separate tubes)	• Reference counting beads • Cell reference standard(s)
Data analysis and reporting	• Number of events collected • Population gating • Underlying assumptions of automated software	• Reference cell FMO (fluorescence minus one) controls • Cell reference standard(s)
Assay performance/ standardization across locations	• All issues described above • Different cytometer operators • Different assay procedures	• Reference materials and methods described above • Standardized procedure(s) • Round-robin study • Training/certification

manufacturers and users toward cytometer standardization. Furthermore, once more traceable standards are available, meaningful correlations of antigen density can be quantitated by all labs.

Very few cell-based reference materials are currently available. One is *CD34+ Cell Enumeration System Suitability* from the United States Pharmacopeia (USP), which has a certified value for CD34+ cell concentration of 16 to 34 cells/μL via flow cytometry (87). This material is made from fixed and lyophilized peripheral blood cells and provides a stable material for CD45 and CD34 staining and serves as an *in process* control for measurements needed for clinical bone marrow and related stem cell transplantation. The other material is the *FITC-CD4 Positive Control Cells* from the National Institute for Biological Standards and Control (NIBSC) with a certified value for CD4+ T cells of 212.1‐438.1 cells/μL by flow cytometry (88). This material contains lyophilized human PBMC prelabeled with a FITC conjugated monoclonal antibody and serves as a positive control for CD4+ T cell enumeration. Furthermore, this material is CE marked for use as an IVD within the EU member states and EEA countries. Because both cell reference materials are made from human blood, they contain clinical analytes of interest and are appropriate controls for detection of cell debris, testing different antibody clones to the same antigen, inter- and intra-laboratory performance monitoring, and training and qualifying new users for their intended use.

Additional cell-based reference materials are needed for counting of other cell types and for quantifying antigen expression in antibodies bound per cell (ABC) (89). The ABC is an instrument independent unit unlike the instrument-dependent MFI. In particular, cell reference standards with well characterized antigen expression (90) are greatly needed for advanced cell manufacturing and cell therapies. NIST and FDA along with industrial partners and user communities are actively collaborating on projects to address these standard needs.

Timely updates on current efforts to develop control materials, documentary standards and methods can be found in WS09 on "Control Cells or Not" reported in the same journal issue, a pending CLSI guideline on "Validation of Assays Performed by Flow Cytometry," and novel computational methodologies for unbiased analysis of complex cytometry data (91). In addition, discussions on antibody quality for

flow cytometry occurred between manufacturers, users, and CYTO meeting management in CYTO 2018. As a result, future actionable steps will be forthcoming. All these activities will ultimately lead to building measurement assurance in flow cytometry (Table 4 and Supplementary Information WS13_SI4).

WS11: Flow Cytometry Application in Multi-Center Global Clinical Studies: The Importance of Standardization and Harmonization

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Introduction and Aims

Flow Cytometry (FCM) is a powerful technique with applications from basic biology and exploratory endpoints to critical safety and efficacy decision-making during drug development. FCM assays should be developed and validated in accordance with recommendations presented in seminal papers (67,92). These support best practices for performing FCM in a regulated environment and are cited in feedback from the experience of cytometrists within the European Bioanalysis Forum (68).

WS11 gave opportunity to present and discuss key aspects considered to be critical to achieving standardization and harmonization of FCM applications in multicenter clinical studies. The attendees were currently involved in, or considering, implementing sophisticated FCM-based assays in multisite clinical testing for global clinical trials in regulated laboratories and among the workshop participants were manufacturers of FCM controls, reagents, and analysis software. Here we report on the survey findings and outcome of the live discussions regarding current challenges and possible solutions. We highlight some of the most advantageous approaches to reduce both the variability and the time required to produce results.

Methods

The online survey and live discussion (Supplementary Information WS11_SI1 for details) were conducted across five categories. The workshop started with a general introduction to the topic (Supplementary Information WS11_SI2), followed by group discussions in each subtopic. Each of the categories, including the live discussions, was handled by one of the WS chairs and the most relevant aspects summarized. Many of the results from the live discussions were in agreement with the online results. In the following sections, we present the main outcomes for each topic and discuss major findings and recommendations.

Results/Outcome

Considerations for specimen type. The most common specimen types involved are peripheral blood and PBMC. Reasons for selection were biological relevance (48.7%), followed by clinical study setup and the ease of sampling/repeat sampling.

Stability is also considered as an important factor in specimen selection (10%). To stabilize blood samples, 49% of respondents rely solely on anticoagulants. Blood stabilizers of different types are used by 40% of respondents.

WS participants identified robust sample transport, low stability of rare specimens, and limited availability of samples from diseased individuals for stability testing as major issues; largely driven from the fact that diseased samples often manifest different behavior to healthy samples.

Challenges and opportunities of local versus centralized analysis. There was an equal separation between those shipping samples to multiple testing sites for processing and acquisition, and those sending their samples to a single test lab (40% each). Fifteen percent of respondents used a mixed model approach and 5% used local clinical site facilities; >70% considered the main advantage of local testing was the ability to overcome limitations in specimen stability, or reduced data turnaround (30%). The top ranked advantage of centralized sample testing was the ability to process samples using the same protocol and measuring on the same instruments. Major challenges for centralized sample testing were considered to be sample logistics and stability (30%), instrument alignment between testing sites (30%) and protocol harmonization for sample processing (22%).

When using local, or multiple analytical facilities the discussion clustered around consistency in 1. personnel (training, experience, compliance with local quality systems); 2. instrumentation (calibration, monitoring, and comparability between different analyzers); and 3. concerns relating to data management and reporting. The use of the 'mixed model' approach was considered to be a pragmatic compromise.

Critical steps for assuring process harmonization and instrument standardization. Standard Operating Procedures (SOPs) were agreed to be the main factor in process harmonization assurance followed by periodical laboratory cross testing (75%). Only 5% ensure between-lab process harmonization by involving robotic/automated sample preparation at each testing location. Nine percent of respondents do not monitor inter-instrument standardization. The competency of technical staff is preferably assessed by internal training prior to or throughout the study, followed by internal blind testing/gating assessment. Only 12% indicated participation in an external Quality Assurance (QA) program.

There is a need for guidelines describing standardization and monitoring of instruments alignment. Users indicated that the procedure for instrument standardization is complex and not well described by producers and more could be done in these areas by the instrument vendors. The majority of participants indicated the importance of well written SOPs and continuous staff training in successful process harmonization.

Control of critical reagents and within study controls in multisite studies. For QC, the majority use beads (41%) and/or stabilized blood (38%) followed by fresh blood and frozen cells for each run (59%) or daily (24%). Presence of all

reagents in the tube is tested after measurements at data analysis stage (67%) and long-term reagent stability is assessed by lot-to-lot comparison (71%).

In the discussion, PB controls were favored over cell lines. Blood could be pooled to reduce inter-individual variance and stored frozen in aliquots sufficient for the whole study period, if stability of the populations and markers allows. Lyophilized samples were not widely used. Control beads are good for standardizing Mean Fluorescence Intensity (MFI) values, but they poorly reflect biological cells (e.g., different light scatter properties) and are only available for one antibody at a time limiting comparison to cells. Concerning standardization in the preparation of critical reagents like antibodies the participants recommended preordering all antibodies and storing them as cocktails for the whole study. Whenever a new cocktail is prepared, it should be qualified for use by overlap assays, tested with stored Peripheral Blood (PB) aliquots.

Standardization of data analysis. The majority of the sites use multiple analysts. More than half of respondents clean their FCS files prior gating. A significant concern was the subjectivity and time associated with manual gating. While the full spectrum of data analysis options were variously adopted, there was agreement that automated methods hold great promise with much to be done in terms of ease of use.

Discussion

Several variables such as sample/specimen choice and preparation, instruments setup and alignment, and data acquisition and analysis impact the outcome and reliability of large scale multicenter studies involving FCM; as identified by the survey and the live discussions. The main outcomes and suggestions from the WS were:

- Control of sample stability (from few hours to several days) is a critical factor in choosing a distributed processing approach and several methods to enhance sample stability are well documented and should be consulted (93,94).
- Lab-to-lab variations in sample processing and analyses were considered major sources of data variability resulting from subjective analyses along with the potential variability introduced by the use of multiple instruments and operators.
- The need for access to disease-state samples for use in assay development and as control material. Suitable samples could come from commercial vendors, clinical partner networks, or the creation of biobanks of disease samples.

Improving reproducibility of analysis starts with the use of standardized gating protocols, encoded in SOPs, gating templates and the use of biological or unstained controls (95). Instrument-associated variability can be addressed through inter-instrument alignment and monitoring (96). This requires detailed knowledge of each instrument performance involved in clinical trial. Even the most up-to-date system monitoring packages for digital cytometers make assumptions, or use calculated models based on one-time (i.e. per day) performance checks instead of real-time characteristics.

Another issue that could not be overcome is that instruments in the same clinical trial have not identical technical details reflected in differences in performance. In this case inter-instrument alignment could be achieved by procedures of harmonization. Both, standardization and harmonization serve to achieve greater comparability of patient test values between different clinical assays. However standardization can be difficult to achieve.

Issues with shipping need to be addressed by better logistics and optimization of sample stability by the use of appropriate stabilization methods. However, a significant amount of variation would still be introduced with center-specific analysis, which cannot simply be mitigated by the use of template gates.

Given the heterogeneity in patient samples and the subjectivity in manual analysis, centralized analysis involving a single analyst has been promoted as an alternative to fixed gating. This concept can be extended by performing all analysis in one batch, and subsequently having a second operator review gates for consistency. However, these options are not scalable to larger studies, due to the time required to analyze each sample. This leads on to the growing use of specialist software tools to reduce subjectivity in reporting (95,97). Automated FCM analysis approaches have reached a demonstrated level of maturity in clinical trials and in the FDA-approval process (98,99). However, it is still important to ensure data is of the highest quality and correctly annotated (20). We consider the use of the "mixed model" approach to be a pragmatic compromise. In this approach, a limited number of regional facilities with appropriated aligned procedures and standardized instruments are used to process and analyze samples. Data analysis and reporting is then conducted via a central controlling facility (95,100). Although currently rarely used by the participants, in future automation could be introduced at all stages of sample preparation (implementation of robotics). The use of lyophilized or dry premixed cocktails would also avoid routine pipetting errors (97,101,102). Samples and reagents could be barcoded, to ease traceability in sample processing and increase the chance of identifying batch defects (101).

There is still a real need for the publication of joint/official guidelines, "harmonizing" the whole process. These should be accessible to everyone involved in multi-site clinical trials. Well-defined pathways for control sample and reagent purchases, handling and storage, as well as for alternatives to cells from healthy and disease-state subjects are crucial in controlling critical reagents. Testing of staining and preparation, as well as the quality of antibodies and cocktails, should be done by biological (external or internal) and full minus one (FMO) controls. We advise that multicolor panels should be developed in standardized manner following the OMIP (optimized multicolor immunofluorescence panel (103)) model where possible. For control of surface antigen phenotyping one alternative could be beads that more closely mimic cells than those presently available. Comparable beads and bead arrays have been in use for characterization of ant-HLA antibodies in serum (104) for some time. Such beads could particularly assist the

testing of antibodies against surface activation antigens that are in low abundance in unstimulated cells and need cell stimulation and culturing. A single bead type could also be equipped with multiple antigens simultaneously. However, these controls come with substantial costs. Alternatively, cell lines that consistently express genetically engineered receptor genes could be useful as positive, or negative controls. In the control of functional and activation assays: such as intracellular cytokine expression of signal transduction, beads are not an alternative for cells, or cell lines.

Taken together, the opinion was that there is still a need for unified protocols and SOPs, to standardize and harmonize all aspects of the flow cytometry analytical process. This includes instrument-independent common protocols, access to test samples from diseased individuals, appropriate/innovative synthetic bead controls and automated data analysis. Once established SOPs should be meticulously implemented across all sites.

Improvements in intra-study QC, unification of protocols between different studies, consistent technical staff training and laboratory participation in external quality assessment programs will all aid improvements in the ability to compare data and increase the value and reliability of FCM-based clinical data (105). Finally, given the complexity of the multicenter study process, collaboration and communication between the clinical and analytical teams is required to generate robust and reliable results to support decision-making.

CHAPTER 3: QUALITY ASSURANCE AND REPRODUCIBILITY CONCLUSION

In summary, there is an urgent need for high quality reference material and documentary standards to reap the benefits from the growing number and complexity of assays performed with multiparameter flow cytometers.

Quality assurance and results reproducibility are of highest importance in clinical studies involving flow cytometry. Unfortunately, many steps of these trials such as sample processing, conditions control, instruments set up, measurements and at last but not least analysis have many weak points. It is quite obvious that there is a sound demand of community members for guidelines detailing sample processing-related procedures and listing control materials employed in flow cytometry-based clinical studies. Before this happens, best practices can be also discussed and experiences can be exchanged by cytometry community members at specially assembled thematic groups, task forces or consortia meetings. In the light of clinical assays complexity augmentation, development of a solid reference document appears to be an urgent necessity.

CHAPTER 4: TECHNOLOGY

Understanding of fundamental biological processes, cellular networking, or drug candidates' proof-of-mechanisms would not be possible without technological advances in cytometry. In this chapter, four workshops discussing technological

developments to aid in cytometric studies were combined. Antibodies conjugated to fluorophores in biomarkers detection and quantification has been used routinely for multisite clinical studies. However, antibodies can be expensive and challenging to work with due to increasingly complex assays and more demanding compensation process. The aptamer technology allows the creation of a single fluorescent aptamer that replace multiple antibodies from a polychromatic panel to detect a specific cell sub-population. Aptamers production can be cheaper, they are easier to handle and more stable alternatives to antibodies (WS08). Time-lapse fluorescence microscopy is another powerful tool in the study of complex biological systems but phytotoxicity, a consequence of excess sample illumination, can adversely affect the sample. This can lead to erroneous and misleading results, but phototoxicity can be mitigated by various measures. These include new developments in microscope technology, software, novel fluorophores, especially moving experimental designs into the far-red range, and the inclusion of proper controls (WS14). Experimental design and consideration of the most appropriate controls to include are an essential part of any study and have been the flash points of many scientific discussions. In multisite studies that include inducible biomarkers, the choice of the proper controls can pose a real challenge as these assays are more complex than the studies of constitutively expressed biomarkers. Inducible biomarker assays require controls for shipment, region and gate placement, and induction of the biomarker. Consensus guidelines are needed and being developed (WS12). Well established and controlled platforms for therapeutic sorting are expected and required in the age of cellular therapies (WS16). However, traditional sorting systems are often not fully enclosed, difficult to operate, and lack the gentle separation mechanisms required for high purity, recovery, and viability. These traditional platforms are being challenged by next generation systems such as microfluidic based systems which are fully automated, sterile, and more efficient.

WS12: INDUCIBLE BIOMARKERS

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Introduction and Aims

Not all biomarkers are present in resting cells. Some biomarkers appear after the cells are stimulated. This can be something as simple as a general activation of the cell or a specific stimulation targeting a well-defined receptor on a subset of cells. Furthermore, turning off cells via inhibitory signals may also lead to appearance of specific biomarkers. The advantage of this type of input/output assay is that it allows the utilization of the cells' functional response as a clinical biomarker, a process that might have much more meaningful clinical relevance.

Requirements for validation of clinical flow cytometry assays have been discussed in several previous publications (92,100,106–110). Inducible biomarkers require more complex

and time-consuming protocols than standard immuno-phenotyping that can pose challenges in validation and application in clinical settings. Additional samples need to be included as controls both for the stimulation and for correct gating of the population of interest (111). Shipping of a sample to a central lab for inducible biomarker testing presents an additional challenge in that even though the cells' immunophenotypes do not change, their ability to respond to a stimulus might be compromised due to travel (94).

The aim of the workshop was to discuss utility, best practices and challenges in designing, testing and validation of flow assays involving inducible biomarkers. Transportation of samples, selection of control cells for stimulation and choices of sample/staining to ensure correct gating were some of the topics discussed. The audience was expected to be scientists from both the pharmaceutical industry and from academic institutions working with biomarkers.

In WS12, we started by discussing different options for transportation of a sample including how much should be done before transportation and how to best stabilize the sample during transportation. We followed with a discussion about how to select and design appropriate controls for the stimulation. The final part was about the selection of relevant controls to ensure correct gating.

Methods

The workshop was divided into three sessions with a total of five online polls using Sli.do (www.slido.com). Poll 1, 3 and 5 allowed only one answer while 2 and 4 allowed for multiple answers (see Supplementary Information WS12_SITable A–E). The number of participants in the workshop was approximately 50 and 24–27 of them participated in each of the five polls.

The first session focused on shipping practices including how much can be done at the clinical site versus the centralized testing laboratory. The session contained one poll and a short overview of validating the use of pre-loaded SMART tubes to allow for both stimulation and stabilization of a phosphorylated protein in whole blood clinical trial samples before shipping.

The second session focused on the use of stimulation controls for inducible biomarker assays. The session included two polls and a short presentation about a case study in which stimulated, fixed and then frozen control cells showed positive signal over background in normal human PBMCs for phosphorylated (p)-AKT/PKB and p-ERK. These stimulated controls, along with their unstimulated counterparts, were critical to the validation of the assay methods and to show the biomarker stability post-draw by flow cytometry.

The third and last session addressed the use of control cells and staining in facilitating correct gating. The session included two polls and a short presentation describing two case studies: i. CD69 expression on B cells after *ex vivo* stimulation of the whole blood, and ii. induced eosinophils shape change assay.

Results: Outcome, Consensus or Similar, Best Practices Proposals

The first topic concerned processing/shipping practices for inducible biomarker samples. From the polling answers, it was clear that most teams favor the route of shipping fresh/frozen samples to a central lab where the stimulation is performed (42%). There are sample stabilizing tubes available that as well as containing stabilization solution, can also be pre-loaded with the stimulant of choice to minimize manipulation at the site; pre-loaded SMART tubes were presented as an example, but this was not the most popular choice (4%) (Supplementary Information WS12_SITable A). Among the audience 17% chose the "something else" this includes variations on the SMART tube concept. Based on the discussion, the audience have had mixed experiences with using SMART tubes for shipping, however most agreed that a solution to this problem would be welcome.

The focus of the second part of the workshop discussion was on the creation and use of cellular assay controls when evaluating inducible biomarkers. According to our poll, most respondents (56%) create their own controls for a particular assay whereas 24% use fresh cells from normal donors as controls. Of the respondents, 89% reported using negative controls for stimulation and 78% reported using positive controls for stimulation (Supplementary Information WS12_SITable B–C). The last part of the workshop focused on the use of controls for accurate gating. The polling questions addressed what types of controls are used for gating with FMO and non-stimulated cells being the most popular. Isotype and healthy controls were used by 35 and 23% of the audience while only 12% are looking for a clear separation of the populations (Supplementary Information WS12_SITable D–E).

Discussion

Stabilization is also a topic of significant interest as can be seen with a large the number of posters presented on this subject at CYTO18. Despite this extensive interest, significant advances in this area are desperately needed (112). Stabilization of the biomarker of interest during transportation depends on the clinical question and is not a one size does fit all type scenario. SMART tubes have been used in several clinical studies (113,114), but based on the experience of our audience they may not be suitable for small volume samples such as those from pre-term babies' cord blood where the cell composition is different and therefore the sample could be over fixed, or where analysis of rare cells is required (due to either specific or non-specific cell loss). Using SMART tubes, however, does mean that all blood cells are present in the sample rather than just a subset, and they overcome the need for extensive manipulation of samples at clinical sites, for example, adding stimulatory agents or preparing PBMC; which can introduce variability, change functional responses and impact on data quality (94).

Working with innovative vendors to solve the stabilization problem will greatly enhance the data quality of our inducible biomarkers, open new opportunities in the choice of clinical sites, extend the range of inducible biomarkers that

can be measured, and increase the impact these data have on moving forward a potential new medicine.

The challenges connected to transportation and stabilization of samples is something that affects all multicenter facilities studies and should be discussed in a future CYTO workshop.

The use of relevant controls is a topic that was continuously discussed, and, over the course of the workshop, many valid points were raised about the use of cellular assay controls as opposed to isotype or FMO controls, especially when used for setting positive/negative boundaries for inducible biomarkers. Inducible biomarker assays require a stimulation step and relevant controls for this step. The vast majority of the participants use negative controls for their stimulation experiments. Some believe that the best control for a marker stimulated in vitro is often an unstimulated sample, stained with the same antibodies as the test sample (115). In fact, some consider this control to be the most important since it allows the level of background to be subtracted from the test samples for improved assay accuracy. In these cases, the unstimulated sample serves as both a control for stimulation and gating.

The positive stimulation control is essential for troubleshooting a negative result. It is not surprising that some leave out the positive stimulation control as it can be difficult to find a stable source, yet some stimulation controls are available commercially. An easily accessible source is healthy donors, but donor availability can be problematic for studies spanning over several years. The harder way is to generate one's own controls. This typically involves isolating PBMC and freezing them down. Once validated it is a stable source of stimulation control cells as demonstrated in a case study presentation.

An important outcome of WS12 and other presentations at ISAC CYTO 2018 meeting is the concept of standardizing portions of flow cytometry studies (WS11) and ensuring that the methods followed are reproducible (WS03). For inducible biomarkers that are often plagued with short-lived positive signals, these concepts are particularly important. Assay consistency may be achieved by the stabilization of cellular transient signals prior to shipping and the use of control cell populations (such as aliquots of well characterized cryopreserved PBMC, preserved and stabilized cells CD CHEX (Streck), Immunotrol cells (Beckman Coulter), Veri-Cells (Biolegend) and WS13) or the use of immortalized cell lines) that can be used to standardize individual flow cytometry runs to ensure the highest quality data is generated.

The challenges involved in designing flow assays for inducible biomarkers compared to assays for static markers is something many scientists struggle with. It is important to discuss it in a workshop forum that allows the community to learn from each other and to move the field forward.

Acknowledgements

WS14: Phototoxicity in Live Fluorescence Imaging—Understanding, Quantifying, and Minimizing It

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Introduction and Aims

Modern fluorescence microscopy techniques offer an unprecedented view inside living biological specimens. However, careful execution is required, not to alter the physiology of the living samples during the observation. Excess illumination causes phototoxicity through generation of reactive oxygen species (ROS) and destruction of endogenous light-absorbing molecules. Most living organisms have evolved mechanisms to deal with a limited amount of ROS produced in the cell. To collect correct and reproducible data, researchers must aim to avoid exhausting the capacity of these endogenous mechanisms. It remains a challenging task to detect when the phototoxicity threshold has been exceeded, as the first manifestations of phototoxicity are rather subtle. Two commonly used, albeit unreliable readouts, are sample morphology changes and fluorophore photobleaching. Even once the phototoxicity in an experiment is recognized, it can prove difficult to significantly reduce the illumination without considerably altering the microscope setup or experimental conditions. Fortunately, strategies to remedy phototoxicity exist and typically involve altering parameters of the illumination. The most effective strategy involves confining the illumination to the focal plane of the detection objective by using light sheet or total internal reflection fluorescence microscopy. Others include increased exposure time, which decreases the peak illumination intensity at the expense of lower frame rate. Pulsed rather than continuous illuminations leaves dark periods for the specimen to recover from the incurred photodamage. Shift to red and far-red fluorophores allows the use of less damaging illumination wavelengths. Optimized detection efficiency (cameras, filters, optical path) allows for lower illumination power. Antioxidants in the media help scavenge ROS.

We organized this workshop because the implications of phototoxicity for live fluorescence imaging remain widely underrated. This issue is acknowledged and has been discussed extensively among microscopists (116–121), also on the pages of *Cytometry Part A* (122). However, with the increased availability of advanced microscopes, the number of scientists using live imaging is growing fast, with many of them remaining unaware of the dangers of phototoxicity. In other cases, phototoxicity is largely overlooked with the fatalistic attitude that it cannot be avoided. This workshop aimed to establish and raise the level of awareness among members of the International Society for Advancement of Cytometry (ISAC) about the challenges associated with phototoxicity. First, we questioned the workshop participants to learn about the existing and perceived barriers to adopting best practices against phototoxicity (instrumentation, time, experimental protocol). Second, we wanted to understand how difficult it would be to overcome these barriers (education, cultural shift,
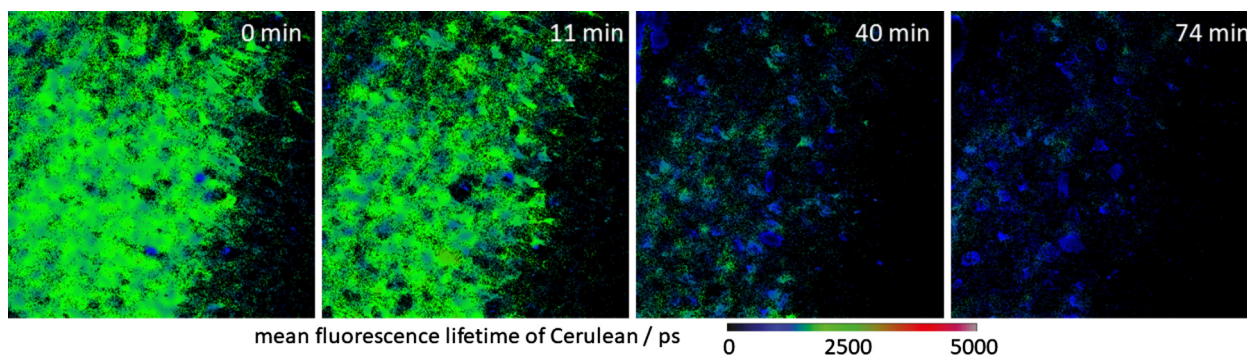
Figure 5. Increasing intracellular concentration of Ca²⁺ during a time-lapse experiment. Although the neuronal morphology does not change, the increased Ca²⁺ concentration indicates phototoxicity. The Ca²⁺ concentration was measured by a FRET sensor TN L15 based on Troponin C, having Cerulean as the FRET donor and Citrine as the FRET acceptor. The shorter fluorescence lifetime of Cerulean indicates a higher FRET efficiency and, thus, a higher neuronal Ca²⁺. The montage shows a brain slice from a CerTN L15 mouse, which expresses TN L15 in several neuronal subsets under repeated excitation (every 30 s) at a mean laser power of 50 mW at 850 nm; pulse width 140 fs. Image size 300×300 μm.

new instrumentation). Finally, we drew experience from the invited expert panelists and the audience to sketch current guidelines for addressing phototoxicity in live cell imaging. The workshop was attended by a balanced mix of students, senior scientists, microscopy facility managers, as well as industry representatives, who all contributed different perspectives in a lively discussion.

Outcome

A number of key challenges that lie ahead were identified during the discussion with the panelists and participants. Overcoming them has the potential to hugely improve the current common practice in live imaging.

Challenge #1: Overcoming the lack of awareness of phototoxicity and the status quo.

In our survey, half of the participants answered that they perform live imaging on a point scanning confocal microscope, which is clearly not optimal from the phototoxicity perspective. More suitable systems are also being employed, as half of the attendees reported using wide-field and 20% light sheet and total internal reflection fluorescence (TIRF) microscopy. Additionally, 20% answered that they do not control for phototoxicity at all. One point that was raised was that even though the general awareness of phototoxicity issues might be good, it is often worse among trainees who are usually performing most of the experiments. Better training from light microscopy facility managers was identified as a key action to improve phototoxicity awareness. Newcomers to the field of live imaging need to be pointed to the recent reviews on the topic (116,117,119) and encouraged to implement the recommendations in practice in their own experiments. Microscope manufacturers were criticized for not providing realistic estimates of what type of live imaging experiments are feasible with their systems.

Challenge #2: Insufficient reporting of live imaging conditions in publications.

Having sufficient information about the live imaging experiment is essential for fair assessment of the reported

data, which currently is not always possible. It would be desirable for scientific journals to adopt a policy of minimal information required for reporting live imaging experiments. Improved reporting would build awareness and eventually establish safe illumination levels for widely used model systems.

Challenge #3: Quantification of subtle phototoxicity effects not evident at the morphological level.

Two thirds of the workshop participants evaluate phototoxicity purely on morphological level, e.g. from membrane blebbing, which can easily result in underestimations (116). Metabolism itself is highly sensitive to any kind of injury including phototoxicity and metabolic changes appear much earlier than any morphological changes, for example, (123). Our panel suggested direct oxidative stress measurement, e.g. of lipid oxidation or increase in intracellular Ca²⁺ concentration, as relatively simple and very sensitive readouts (Fig. 5, references in (116)).

When imaging mammalian embryos, a more complex but relevant way to measure phototoxicity is to reimplant imaged embryos in the carrier mothers as has been done for two-photon (124) or light sheet imaging (125). Their development to pups has been followed in comparison to non-illuminated fetuses. Similarly, the Beaurepaire lab investigated the development of *Drosophila* embryos after excitation between 1 and 1.2 μm (126).

Challenge #4: Lack of commonly available hardware and fluorophores optimized for imaging in the far-red spectrum.

Shifting excitation toward the red and far-red wavelengths alleviates phototoxicity and decreases background fluorescence from the absorption by endogenous molecules (116). Reducing background fluorescence should be viewed as an equally powerful strategy to improve the image contrast as is increasing the signal (121,127). There was a consensus that switching to longer wavelengths offers a considerable potential for reducing phototoxicity and indeed, half of the participants are already doing that. Still, half of the participants reported that they use GFP constructs out of habit or because their

Table 5. Measures to alleviate phototoxicity in fluorescence microscopy and appropriate control experiments

ACTION	POWER TO REDUCE PHOTOTOXICITY	CHALLENGES
Imaging media additives	+	The experiment is performed in conditions different from the standard in the field, for example, in hypoxia when using oxygen scavengers
Extending exposure time and reducing illumination intensity, pulsed illumination	++	Not suitable for very fast processes
Using far-red fluorescent probes	++	Detection components have lower efficiency in that spectral range, far-red fluorophores have lower brightness
Using selective illumination, for example, light sheet	+++	Need for potentially expensive hardware and new sample mounting strategies
Using deep learning for image restoration	++	Currently requires advanced coding skills

Potential controls for phototoxicity
- Include a transmitted light channel to monitor sample morphology during imaging.
- Monitor sample health after finishing the experiment, e.g., if cells divide, or embryos hatch.
- Compare a nonilluminated to an illuminated (part of) sample at the end of the experiment. Check for slowdown of the cell cycle (lower percentage of mitotic cells), cellular membrane blebs, delayed development of embryos, etc.
- Generate the phototoxicity (dose–response) curves (Tinevez et al, 2012; Icha et al, 2017; Schmidt et al, 2017) for novel experimental setups.

trusted constructs are GFP fusions, citing historical reasons or technical limitations. Most live imaging setups are optimized for GFP, as detection components have often poor efficiency in the far-red spectrum. Microscope manufacturers should reflect the needed shift toward longer wavelengths and bring appropriate live imaging setups to the market. Development of better far-red fluorophores and detectors is a field of active research and will keep providing improvements in the future.

Challenge #5: Synergistic effects of cellular stress and phototoxicity.

Phototoxicity assessment is often performed on unstressed samples, which does not reflect the usual experimental situation of suboptimal culture conditions or inhibition/knockout of important cellular proteins. For example, the capacity of cells to scavenge ROS is reduced when they are metabolically challenged. It is therefore essential to consider that the more stress the experimental conditions impose on the cells, the sooner and more likely will phototoxicity occur.

Challenge #6: Introducing the low O_2 concentration imaging.

Imaging of mammalian cells is typically performed at near-atmospheric O_2 concentration, which is much higher than its concentration inside the tissues in vivo. At the same time, it is known that lowering O_2 concentration can reduce phototoxicity and photobleaching (128,129). Despite these obvious benefits, low O_2 imaging is rarely performed. This is mainly because maintaining the cells in hypoxic conditions is technically demanding and such experiments generate data that are not directly comparable to the body of existing literature. Thus, it remains to be seen whether the live imaging field will move in the direction of low O_2 imaging.

Challenge #7: Reducing phototoxicity in multi-photon microscopy.

In the case of imaging deep inside living tissues or organisms, multi-photon microscopy remains the method of choice. In multiphoton microscopy, both the processes of photodamage and photobleaching at the focal plane follow a highly nonlinear dependency, even higher than the excitation itself (130). Reducing the repetition rate of the lasers, which enables a longer time for the cells to recover before the next excitation (131) and pushing the excitation further to the infrared and the emission further to red or even near-infrared are suitable strategies to reduce phototoxicity (132–134).

Challenge #8: The use of image reconstruction software algorithms for enhancing image contrast needs to be streamlined.

Recent publications using deep learning for reconstruction of fluorescence microscopy images (135,136) hold huge promise for the future. Such algorithms can reconstruct high quality images from noisy raw images acquired at low illumination levels. Currently, using these algorithms is not straightforward and their implementation for reconstruction of individual raw data-sets needs to be streamlined. We expect continuing rapid development in this area, which will make these algorithms accessible to a wide community of biologists.

Perspectives

The overall atmosphere at the workshop was optimistic. Participants agreed that we no longer have to nor should accept phototoxicity in our experiments. Technological advances like light sheet microscopy, improved scientific Complementary metal-oxide-semiconductor (sCMOS) cameras, new fluorophores, and so forth (see Table 5) were

identified as the most important recent developments. Their cross-disciplinary nature however requires synergy between biologists, engineers, physicists and computer scientists. The trend of moving toward live imaging and the need for more training in image processing is also apparent from a large community survey in the recent Biotechnology and Biological Sciences Research Council (BBSRC) report on bio-imaging in the United Kingdom (137). Triggering some phototoxicity should not stop us from pushing the applications at the frontier, as long as appropriate control experiments are conducted to monitor sample health (see Table 5). In addition, increased understanding of phototoxicity mechanisms and effects can be applied beyond imaging, for example, in photodynamic therapy or to trigger precisely localized DNA damage. Another outcome of increased awareness related to phototoxicity is that scientists and editors are becoming more rigorous and demanding when reviewing papers and judging live imaging data. This will contribute to improving reproducibility and quality of information in the literature. A refreshing suggestion from one workshop participant was that the live imaging community should crowdsource their own phototoxicity guidelines through GitHub, similar to how the biomedical deep learning community reviewed the current state of their field ((138) github.com/greenelab/deep-review). The consensus was that since the topic is so broad, several parallel guideline projects would be needed for specific fields and microscopy techniques. Additionally, there was an encouraging interest from company representatives present during the workshop. Hopefully, new light microscopy products with the purpose of controlling and reducing phototoxicity in time-lapse imaging will become available in the near future.

Acknowledgements

WS08: Aptamers in Cytometry

Henning Ulrich, Matthias Schiemann.

Aim and Introduction

The aim and justification of this workshop was to introduce and discuss with researchers the process of aptamer development and fluorescence-tagging for the use in flow and imaging cytometry applications. RNA and DNA aptamers are developed by an in vitro selection process, Systematic Evolution of Ligands by EXponential enrichment (SELEX). This technique developed in 1990 parallel by Larry Gold and Jack Szostak (139,140) is based on screening of a target molecule against a partially random RNA or DNA library. This library consists of an inner random region, flanked on both sites by constant regions for primer binding for RT-PCR or PCR amplification. Reiterative cycles of target binding, elution of target-bound and discarding unbound oligonucleotides are followed by RT-PCR or PCR amplification. Five to twenty cycles may be needed in order to purify the random library of approximately 10^{12} different sequences to a homogenous population of high-affinity target binders. From this final pool, aptamers are identified by sequencing and grouped into classes, based on sequence similarities within the inner region, which consisted of random sequences prior to the onset of selection. Aptamers are individually tested for binding activity. Target selectivity is tested by comparing aptamer binding efficiencies with those to targets with similar structures. Exposure of the library against undesired similar target molecules and discarding of DNA / RNA molecules binding to them can be used to enhance selectivity of the selected aptamer library. The best aptamers are modified for their respective applications, such as for instance imaging and flow cytometry (see Supplementary Information WS08_SIFigure 1 for DNA aptamer development). Fluorescence-tagging of DNA aptamers can be achieved by PCR amplification of aptamers in the presence of fluorescently labeled primers and gel purification of the labeled single DNA strands.

Aptamers have been selected against a wide range of targets including small molecules, peptides, a great number of proteins, including growth factors, enzymes, receptors, erythrocyte ghosts, virus, bacteria, parasites, stem and cancer cells (142–144).

Recent improvements have been made in oligonucleotide synthesis, making aptamers more stable and reducing costs of their development. Advances in selection and screening protocols allow aptamer development for multiple targets. Aptamers comprise a novel class of high-affinity and specific ligands for cytometry and in vivo applications for unveiling mechanisms in basic science, diagnostics and therapy (145,146). Aptamers rival antibodies in diagnostic applications, due to their synthetic nature, low cost production based on advances in oligonucleotide synthesis, stability and easiness of modification, such as the attachment of fluorescence probes (145–148). A novel application, denominated Cell SELEX, explores the expression of cell surface epitopes that differ between two given cell types or between healthy and disease cells. Using cells as targets, aptamer libraries can be identified that bind to biomarkers expressed by target cells and not by any other cells (140). Aptamers have been developed that specifically interact with cell surface epitopes of parasites (trypanosomes or malaria-infected erythrocytes) or distinguish between the differences in molecular signature of somatic, stem and cancer cells (141,142,149,150). Aside from their use for target cell identification by image and flow cytometry and laser-scanning microscopy, aptamers can be used for isolation of target cells from complex mixtures by cell sorting.

The audience of the workshop consisted of researchers, students, professionals and antibody vendor representatives who use flow cytometry and were interested in aptamer development. The goal of the workshop was to familiarize the audience with planning and execution of aptamer experiments and

to confront and seek solutions to limited selectivity and low affinity of aptamers.

Methods

A slide-show introducing aptamers use in cytometry was alternated with an interactive discussion with the audience. Questions were made by the chairs and discussed with the audience; then, specific questions by the audience were discussed with suggestions of the chair. Questions on specific applications and limitations were put for discussion of the audience. Perspectives in novel biomarker definition, tumor detection, molecular profiling of a large number of biomarkers in blood samples by aptamers were discussed. Features of aptamers were emphasized, such as that they can be developed for almost any targets or differences in the cell surface between cell types (i.e. tumor cells vs stem cells). Discussion points are listed in the Supplementary Information. The major points of the discussion were annotated.

Results/Outcome

The workshop aims were met by raising the audience's interest and understanding of aptamers and their applications in cytometry.

New ideas and concepts were created and discussed during the workshop, encouraging new research and company projects, as detailed in the Supplementary Information. The opinion of the audience was that the use of aptamers in multiplex fluorescence labeling should increase in the near future, such as already shown for aptamers in proteomics applications (151). The production of aptamers may be cheaper, their modification easier and their target binding affinities comparable to those of antibodies. Most importantly, aptamer selection can be done in any laboratory with basic molecular biology equipment.

Discussion

This workshop consisted of educational aspects on the use of aptamers in cytometry and the development and discussion of concepts and applications with the audience. The new improved products (aptamers with enhanced selectivity profiles and stability in biological fluids) are synthetic, high-affinity ligands coupled to fluorophores for flow and imaging cytometry. However, the complexity of the technique requires adaptations for every target epitope or cell, such as aptamer binding and amplification protocols during SELEX and post-SELEX modifications for diverse applications. A second workshop is necessary with more detailed discussion of protocols focusing on critical steps and possible draw-backs. Further, protocols published on aptamer selection for flow cytometry (i.e., ref. (143)) need to be updated with the latest developments, i.e. aptamer-facilitated mass cytometry used for characterization of leukemia cells (152). Aptamers, which can be selected in parallel against a variety of targets for multi-panel studies (153), will benefit researchers. In summary, aptamers have advantages over antibodies: 1. they are developed in vitro and can be raised against almost every target; 2. they can be truncated and optimized and stabilized by chemical modifications; 3. lots do not differ in their activities, as they are produced by chemical synthesis; 4. aptamers have desirable storage properties, as they can be renatured after denaturation; 5. they do not provoke immune responses in in vivo applications; and 6. aptamer development and production costs are lower compared to antibodies.

A major advantage of aptamers is that a single fluorescent aptamer can be developed to replace multiple antibodies of a multicolor panel to detect a specific cell subpopulation. This minimizes compensation challenges in a multicolor panels in flow cytometry and opens new areas of detection possibilities. Replacing multiple antibodies by a single high-affinity aptamer is particularly beneficial to cell sorting, where it vastly reduces time, complexity and cost of magnetic- and fluorescence-activated cell sorting (MACS and FACS).

The main limitation of aptamers is the lack of commercial, readily available epitope-specific aptamers, while antibodies for a large number of epitopes can be purchased. However, custom development of specific aptamers is commercially available. Aptamers are commonly negatively charged, conferring low affinity binding to positively charged epitopes on cell surfaces. The partial disadvantage of low binding affinity of some cell surface epitope-binding aptamers in comparison to antibodies, for example, in flow cytometry applications could be overcome by using aptamers multimerized aptamers with combined avidity.

Conclusion and Perspectives

Based on the presented material and discussions during the workshop, we conclude that the audience (cell biologists and cytometrists) became aware of aptamer development approaches and applications, including their benefits and weaknesses compared to antibodies. Aptamers are an alternative to antibodies in cytometry applications due to their robust nature, flexibility in accessing binding sites and low production costs. Researchers should be aware of possible aptamer binding to unspecific sites as result of negative oligonucleotide charge.

Acknowledgments

WS16: Therapeutic Cell Sorting

Christopher J. Groves, Lina Chakrabarti, Michael Lee, Stefan Radtke, John Sharpe, Samson Rogers, Grace Chojnowski.

Introduction and Aims

Fluorescent Activated Cell Sorting performed by droplet cell sorters (with either a jet-in-air or cuvette-based laser/stream

intercept) has been an essential technology for the identification and isolation of cells in research, clinical, and manufacturing settings. However, conventional droplet sorting presents a number of challenges, particularly in Good Manufacturing Practice (GMP) settings. Medical research has provided transformative applications for sorted cells, particularly in cell therapy of cancer (154). Since the first CYTO therapeutic sorting workshop in 2014, new sort technologies specifically developed for therapeutic cell sorting are now commercially available. Microfluidic cell sorters, in particular, have emerged as a sorting approach offering solutions to challenges faced by conventional cell sorters (155). The collective experience with this technology is limited, so a major goal of the workshop was to familiarize attendees with the new systems as well as share collective experience toward formulating best practices for GMP sorting.

Methods

The format of the workshop was comprised of six 7–10 min presentations by different facilitators in an open dialogue allowing for attendee participation (Supplementary Information WS16_SI1). The presentations were comprised of:

1. Cell sorting for Biomanufacturing
2. Cell sorting approaches for immuno- and gene-therapy
3. Microfluidic cell sorter development and capability

We prepared a pre-conference survey questionnaire (Supplementary Information WS16_SI2) to address each of the four key areas: 1. sample through-put and obtaining sufficient numbers of cells; 2. sterility and safety; 3. ease-of-use; and 4. cost to facilitate discussion. We created a survey (156) link and posted to the Purdue Cytometry email list (157), and local user group lists before the workshop to collect information from cores currently pursuing the method. We received 36 responses from around the world.

Survey results were discussed at the beginning of the session followed by presentations and general discussion. The workshop ran long by at least 20 min due to the presentations and active and frequent audience participation. The challenges of cell therapy provided by this report are concluded through the presentations (Supplementary Information WS16_SI1), the inclusion of preconference survey (Supplementary Information WS16_SI2), and live discussion which was combined to generate a report for unmet needs.

Results of Survey

WS16 was held on 30th April 2018. Approximately 90 users attended the meeting. Attendees were comprised of scientists and clinicians from academia, Biopharma, and clinical labs. Many participants completed a pre-workshop survey (Supplementary Information WS16_SI2). The point of the survey was to seek consensus answers to frequently asked questions and provide information metrics for laboratories and groups thinking of setting up GMP capability or service.

Sample through-put and obtaining sufficient numbers of cells. Eighty eight percent of the respondents indicated that cell throughput of their platform/instrument is very to extremely important. The types of applications employed by respondents were equally distributed between cell therapy, bone marrow transplantation, cell manufacturing, and other. Ninety one percent agreed that starting cell numbers and overall cell throughput of their cytometer was a main concern. Eighty eight percent of responders indicated they use only one cell sorter to perform their applications and do not spread out their sorting on multiple instruments. Forty percent of respondents used automated systems or kits for their cell preparation or separation, and Ninety percent agreed or strongly agreed that automation is essential to their application process.

Sterility and safety. Ninety percent of respondents ranked sterility and/or safety as an important factor to their process. To address the potential for cross contamination of specimens, most respondents replied that they rinsed/washed sample line between samples and the most common cleaning method was with bleach, detergent, and water. The respondents were equally distributed between ISAC biosafety guidelines, internal/proprietary SOP and a combination of the above for the determination of best practices to ensure cell sterility. Ninety eight percent agreed and 68% strongly agreed in the need for a specific guideline for cells sorted for therapy, manufacturing, or transplantation.

Ease of use. When asked about preferred systems most respondents ideal platform would be easy to very easy to operate. Eighty six percent of respondents indicated that they had a minimal level of qualification or certification for operator/s in their labs. Sixty percent of respondents used commercial compared to in-house or proprietary reagents for cell labeling. When asked to rank the importance of GMP/GCP or animal component free reagents, 60% indicated it was an important factor. Seventy five percent of respondents used less than six parameters when examining and selecting cells for purification, indicating a relatively simple instrument design is needed by most labs.

Cost. While most agreed that cost was an important factor in choosing the optimal sorting platform, only 16% strongly agreed. Since most current cytometer platforms do not have disposable fluidics, 60% of respondents indicated they did not use pre-sterilized fluidics and flow cell components. Nearly 64% of respondents indicated that the cost of disposable components presented a hurdle to adoption or use of new methods in their labs. When asked if it was available for their platform, would they purchase irradiated/sterilized single-use fluidics components even at a high cost, 38% responded they were likely to very likely use them.

The results from the discussion were largely in line with the survey results and consensus from the audience was similar regarding new platforms needed which were more fit-for-

purpose. Comments and concerns for FDA qualification requirements among the debate was left unanswered.

Discussion

The workshop was intended for anyone interested in sorting cells for cell therapy, transplantation, or therapeutics manufacturing. The assembled speakers discussed their current work with GMP sorting highlighting the benefits and current limitations of these systems with special attention paid to the qualities of microfluidic systems being developed. Topics addressed by the pre-conference survey were discussed during presentations focused on the difficulties in GMP cell sorting. Experiences and approaches to therapeutic manufacturing and cell therapy sorting across current instrument platforms were discussed between the panelists and audience. The intention of the workshop was to identify uses and issues related to the cell sorting technology and methods.

The main critical regulatory emphasis in biomanufacturing is that the manufacturing cell line must be derived from a single cell progenitor or clone following transfection and media adaptation. In addition to assurance of clonality of manufacturing cultures, the sterility of cell sorting is of paramount importance, ensuring that there is no carry over or contamination from one producing line to another. Using the BD Influx® (158) cell sorter with single-use disposable gamma-irradiated fluidics components to deposit single cells into culture plates, followed by Cellavista® (159) imaging, provides assurance of monoclonality and satisfies regulatory requirements (160). This method contributes to a cost and time saving approach yielding high single cell deposition efficiency and post sort viability, leading to 50% outgrowth. However, low cost, easy to use alternative technologies with gentle processing (low pressure and shear stress) could potentially amplify the savings on time and resources.

BD FACS Aria (161) flow cytometers, located in a clinical GMP suite, are currently being used to isolate regulatory T cells for expansion and adoptive transfer to patients. To perform this process, operators employ rigorous sterility and cleaning measures and benefit from semi-automated setup and quality control measures. However, the high cell yields needed for this work are performed one patient at a time, due to concerns for contamination. Therefore, while droplet sorters collect populations based on a myriad of selection markers, aseptic sorting method preparation along with the expense of the instruments relative to sample throughput are limiting factors that could be improved with fit-for-purpose instrumentation.

Targeting Hematopoietic Stem Cells (HSC) with gene therapy has vast potential to cure genetic diseases. However, the current state of the art for the isolation of HSC requires both multiparametric cell sorting and cell manipulation with sophisticated ex vivo gene transfer conducted within a dedicated GMP facility, limiting availability of this method to fixed sites (162). Unlike droplet sorters, CliniMacs® (163) bead sorting systems can be operated as closed systems, effectively allowing for semi-automated GMP-like cell enrichment without the need for a specialized facility. While bead-based capability can be limiting, when combined with a Tyto® (164) microfluidic cell sorter, a more complex cell phenotype may be collected than by the bead approach alone. In the case described, the combined methods improved cell recovery by >50% compared to droplet sorting systems without requiring specialized staff. Particularly notable for the Tyto® (and likely for other similar systems) was the isolation of multiple cell subsets from small specimens by employing repeated rounds of selection sequentially performed on the unsorted fraction.

Microfluidic cell sorting devices continue to be developed for low cost, ease of use, and ultra-sterile disposable components. Cellular Highways® (165) has invented a new microfluidic cell sorter based on an inertial vortex, which is being tested for high-throughput GMP cell sorting. The device has a high switching rate and a tiny footprint, potentially enabling parallel channel sorting, and enhanced cell processing rates. Systems like the Gigasort® (166) use multiple parallel fluidics channels with specialized optics to allow cell selection rates comparable to droplet sorters (167). These microfluidic systems generally operate at pressures much lower than droplet sorters which may help reduce cell stress incurred during the purification process (168). In addition, fully enclosed systems like the Tyto® and Gigasort® and small portable systems like the Wolf® (169) allow for biocontainment and sterility assurances and portability, which was presented and discussed as a key aspect for cell therapy sorting.

Attendees left the workshop with a better understanding of the benefits of current and near future instruments as well as the limitations in the field that still need to be addressed. The discussion was lively with both the panelists and audience posing thought provoking statements and questions. Common themes emerged around actionable solutions which focused on optimized platforms needed in order to improve these capabilities. The workshop survey highlighted current concerns for therapeutic product cell sorting. Contamination risks of systems in GMP cell sorting facilities has long been appreciated as a weakness in the use of droplet sorters; indeed, it drives the second highest concern, cell through-put, which is largely constrained by the need for long processes to ensure contamination is controlled. The current generation of fully enclosed systems is finally starting to challenge traditional systems in terms of providing improved ease of operation, disposable and sterilized components, and gentle separation mechanisms. Cell sorting options for GMP settings have come a long way since the first workshop. New purpose-built systems to implement the practice of innovative and potentially curative cell therapies are waiting for their turn.

Conflict of Interest

CJG and LC are full-time employees and shareholders of MedImmune/AstraZeneca. JS is a full-time employee and shareholder of Cytonome/SST LLC. SR is a full-time employee and shareholder of TTP plc.

CHAPTER 4: TECHNOLOGY CONCLUSIONS

In summary new technologies are emerging in the field of biomarkers detection, therapeutic cell sorting and time laps imaging microscopy. However, there is a need from the community for open discussion, free information flow and official guidelines on proper process controls.

Increased technology development allows scientist to understand in more details inter-, and intracellular processes, as well as to define molecular mechanisms underlying many biological phenomena. It also enables the design of targeted and efficient disease treatments. New technologies must be accompanied by appropriate, reliable control measures that will allow scientists to draw pertinent conclusions form their experiments. Finally, successful new technological development requires that, dynamic scientific dialogue involving experts from various disciplines should be maintained at all stages of its development.

FINAL CONCLUSIONS

Trends

There is a strong trend in the development of single cell technologies such as mass cytometry and spectral cytometry with measurement of a higher number of parameters and with better accuracy. These technologies are still lacking unified procedures regarding sample preparation and complex data processing. Another emerging field is genomic cytometry that goes far beyond protein quantification and imaging and enables researchers to gain new insights into single cell characteristics that can be further employed by initiatives such as Human Cell Atlas or cell Omic technologies. Since genomic cytometry is still in its infancy, a lot needs to be done in order to establish appropriate technology related procedures such as sample preparation.

Finally, the trend of initiation and maintenance of dialogue between various community members gathered around specific subjects by building interactive forum, web-based database of knowledge is thought to be the driving force for innovation in the field of cytometry.

Shared Research Laboratory (SRL) Best Practices

SRLs are expected to provide high quality service to the end users offering them the newest technical developments and scientific background allowing for sample measurements, data processing and counseling. This requires time and financial investments in instruments, talents acquisition and retention. Also, there is a need for exchange of information within the community regarding sample processing related procedures, and software for SRL efficient management.

Quality Assurance and Reproducibility

Clinical studies require official guidelines regarding instruments standardization, process harmonization and quality control materials to be used in study. Even though many attempts were made in these fields still there is a lot to improve. In order to establish procedures and decide about high-quality control materials open discussion within clinical

cytometric community, regulatory bodies and instrument/reagents manufacturers must be continued.

Technology

There have been numerous advancements of technology in cytometry, like development of aptamers, a potential alternative to antibodies, employment of technical solutions to counteract the phenomenon of phototoxicity induced during time-laps microscopic measurements such as sCMOS technology cameras or use of far-red fluorophores. In studies involving inducible biomarkers, a discussion on the need of guidelines regarding procedures and controls has been initiated. In the field of therapeutic sorting new, fully automated, sterile, and more efficient platforms have been developed to offer lowered contamination risk, improved ease of operation and gentle separation mechanisms.

Final Remarks

In summary, this first joint report of the CYTO2018 conference workshops was a challenging and so far, unique endeavor intended to provide the global community with the latest information on ongoing trends and developments in single cell analysis as well as to inform about critical and yet to be solved challenges in Cytometry. By nature, such a report summary provides important insights into what is going on but cannot claim to comprehensively report all discussion details and aspect of an individual workshop. Voice or video recording was not feasible in most cases so some detailed information could have been missed. Size restrictions of this joint report also might have truncated some parts of the discussions. In such cases we encourage the reader to directly contact the workshop authors for more information.

This project is a prototype, so far unique and surely will leave open questions for discussion. Thus, we hope for critical feedback from the readers in order to evaluate this format and to improve future CYTO workshop summary reports.

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