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Title: Challenges in transitioning from 5 color to 10 color flow cytometry.

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INTRODUCTION

Flow cytometry is a robust technology that can be used for several applications in oncology, hematology and immunology. The instrumentation for clinical applications has evolved from 2 color to 4 color and now 10 and 12 color. Developing a new 10 color assay or converting an existing 5 color assay into 10 color is appealing on many fronts, however more colors adds more complexity. Therefore, this process needs to be well thought out and designed with input from all affected stakeholders. A team of stakeholders should be assembled, and the following questions answered:

1. Why do you want to move your assay to a 10 color format?
2. What process will you use?
 - a. Sequential, simultaneous or hybrid?
 - b. Screening and add ons?
3. Who will be involved in this process?
 - a. Who are the decision makers?
 - b. Who will perform which functions of the process?
4. What are the stakeholder requirements of the panel?
5. How will the end goals be accomplished?
6. When do you expect completion? Define realistic timelines for each step.

Detailed discussion of each of these questions will follow.

The authors each have different positions and affiliations; and enthusiastically embarked on this transition. This document will share some of their experiences and words of wisdom. Note that since this guide is meant to generate questions regarding how one plans a transition from a 5 color (or less) to 10 color, flow cytometry laboratory specific technical details will only be mentioned in passing where appropriate with references made to technical guidelines from the ICCS or in the literature.

Why do you want to transition to 10 color flow?

The decision on why transition to 10 color flow is of utmost importance and affects subsequent decision making. While 10 color flow has substantial advantages in specimen and operational efficiency (e.g., more antibodies per sample and fewer redundant antibodies) these advantages must be weighed

against the increased complexity of these assays and in certain applications such as routine CD4:CD8 counts, 5 color flow may suffice. Answering this question for your laboratory requires input from the main components in your laboratory; namely technical/development, operations, and medical.

In many (smaller) laboratories, the roles of development, operations, and medical are merged with overlap within individuals but essentially this model breaks down the laboratory section into:

1. A technical/development component that designs, validates and implements new tests
2. An operational component that runs and manages the day to day operation of the laboratory, which includes budget and service metrics such as turn-around-time and QC.
3. A medical component (the medical director(s)) that determines the needed test performance and capabilities.
4. Other stakeholders that may need to be consulted ad hoc are: reimbursement and billing, logistics, and Quality Assurance.

In some large reference laboratories and hospital settings, these three stakeholders may have separate infrastructures and management. In this setting, it is critical for all stakeholders to discuss each other's requirements early in the process.

The interplay and requirements of these components are the starting point in any 10 color transition and when communication between these sections is lacking, poor results will ensue. For example:

- Technical
 - It is extremely important that the assay be completely optimized before validation begins. If it is not optimized, the validation already performed must be repeated, increasing cost and frustration.
 - Technical/development's design of the tube may not take into account the performance needed (e.g., low level detection/sensitivity limitation due to spillover) for that particular panel.
- Medical
 - The medical director may desire CD30 as an additional marker for T cell neoplasms, however since internal positive controls are usually lacking, an external cell line control will be required to monitor for the assay performance with significant operational (time and reagent) costs
 - The medical staff may want to include a number of newer diagnostic and prognostic markers, though this could add significant cost to the test which will not be reimbursed
- Operations
 - Operational desires to decrease add-on testing by performing markers upfront for common entities. This leads to increased add-ons for more critical entities (e.g., LGL populations are common and of little clinical significance however other T cell neoplasm are rare but cause significant morbidity and mortality)
 - A triage screen may be used to screen out normal cases and add on markers customized to the case, which may increase turn-around-time and add staffing requirements.

Vignette 1:

In one example of a transition from a 5 color to 10 color leukemia/lymphoma panel, the laboratory administration was looking to reduce the cost/test of the leukemia/lymphoma flow cytometry assay due to reimbursement cuts. It was important not to adversely impact turn-around-time or quality. A new instrument was purchased and installed and pressure to transition and recoup the investment was high. Operational technologists were assigned to develop and validate the new panel that was designed by the medical staff. The medical staff was interested in using novel diagnostic and prognostic markers though reluctant to give up the other markers in the previous panel. The proposed panel was 30 markers instead of the original 24 markers. They paired the markers together so they would facilitate lineage definition. The operation technologists purchased the reagents defined and began the validation with samples they had procured from another lab. The technologists did not have expertise in panel design and frequently were pulled out of the validation to work on the testing bench. The validation data once completed did not look well because the antibodies and fluorochromes were not paired appropriately. The comparison values were discordant, and, in some cases, results missed the predicate diagnosis. Much time and resources were spent before the project had to be started over.

Vignette 2:

In another example of a transition from a 5 color to 10 color platform, the laboratory administration wanted to both expand testing to AML MRD which could not be economically (or technically) done on the existing 5 color platforms and achieve operational efficiencies from switching from 5 color to 10 color panels. Neither the development nor the medical staff had experience with MRD analysis and the specific requirements thereof and they approached the panel development with similar panel designs to leukemia lymphoma analysis not aware of the specific needs to map out maturational continuum in disease processes like AML MRD. The project was eventually shelved after a year of struggles. Likewise, the switch from 5 color to 10 color L/L panels recapitulated the nearly 10 year old 5 color panels without: 1) updating for new advances and recommendations and 2) maintaining sufficient redundancies between panels to best characterize smaller populations. Furthermore, these new panels were implemented as large lot buys of DuraClone reagents limiting the ability to rapidly redesign these panels once the flaws were noted resulting in excess custom panels with insufficient redundancy (and additional cost in reagents and labor) to phenotype small populations.

All these scenarios can create significant work and subsequent rework once problems are encountered. This can delay the launch of a robust 10 color assay and/or create problems that could impact customer satisfaction and patient care.

In addition, expectations of stakeholders (operational, medical, administration, technical) must be taken into account and managed. Such expectations include

- Length of transition - dependent on many factors: 1) experience - consider hiring or contracting for staff with experience in 10 color flow before or during the transition, 2) case volume - high volumes provide plenty of material for validation, 3) multiple revisions of panels - initial design may (will) not be adequate for your needs if this is your first time designing 10 color panels
- Increased Turn-around-time - unlikely to see significant improvements in TAT compared with 5 color

- Decreased cost - at most 10-15% in most labs (decreased reagent use and fewer tubes set up) compared with relatively fixed staffing costs and increased technical costs
- Increased performance in rare event detection and low cellularity specimens - while 10 color certainly will improve the low-level performance of MRD assays, preanalytical factors such as specimen hemodilution or paucicellularity predominate as causes of suboptimal results and no flow cytometer in the world will save you from that (i.e., garbage in/garbage out)

The reality of 10 color flow cytometry is that most practitioners should choose to prioritize increased performance of the assay (increase sensitivity by collecting more events, looking at more markers of aberrancy, improved/more robust gating strategies) over operational considerations. However, increasing the performance of one's assay is a continuous process (continual process improvement) which starts with the recognition that no panel is perfect, that technology improves, and as new fluorophores become available, they should be evaluated and used to replace existing combinations to improve signal-to-noise and allow for easier discrimination of positive and negative populations. Some examples include:

- ECD/PE-Texas Red to PE-CF594
- PerCP-Cy5.5 to PeCy5.5.
- Brilliant Violet type dyes in the place of Pacific Blue, Krome Orange, or AmCyan

Ultimately, while most panels are under-optimized, they will suffice for 95% of cases, but in the authors' experience, you will wish you had the extra performance, extra separation, or cleaner background on a difficult case at 5pm on Friday night. It is in those situations that your labors will pay dividends.

Managing the operational considerations of a 5 color to 10 color transition

Transitioning a lab from 5 color to 10 color flow cytometry is no easy feat; when coupled with the reality that the lab is a mission critical facility that cannot go down for any extended period of time; this becomes as much a logistical challenge as a technical one almost akin to beating heart surgery. There are two principal models for transition: 1) sequential platform then panel transition or 2) simultaneous platform and panel transition. The description, pros, and cons are:

- Sequential transition
 - Gives the lab time to adjust to new instrumentation before running new panels
 - More spread out operational timeline, platform transition can happen months before panel transition
 - May not be possible if the 10 color instrument is not configured to run your 5 color panel (e.g., PeCy5 vs PeCy5.5 on a FC500 to Navios transition)
- Simultaneous transition
 - May be your only choice
 - Requires machine validation and panel development or validation to occur simultaneously
 - Major logistical challenge to shift and validate instrumentation
 - Almost unheard of in a large lab
- Hybrid transition
 - Running both 5 color and 10 color platforms
 - Running triage panels as 5 color and add-ons as 10 color or vice versa
 - Requires an increase in precious laboratory bench space

- Increases reagent utilization

Changes to add-on strategies in a 5 color to 10 color transition

It should be obvious that an efficient add on strategy requires a thorough understanding of the expected case mix at your laboratory. For example, an institution that sees a large incidence of CLL may wish to run diagnostic/confirmatory markers for CLL/SLL (e.g., CD200/CD23/FMC7) upfront in their diagnostic strategy whereas institutions with a lower incidence of CLL (perhaps a lab that supports a pediatric hospital in addition to the general population) may wish to reflex to such markers in the interest of costs. The retrospective analysis of case distribution should be performed rigorously rather than on the basis of a gut feel and experience since in my experience there is a marked recall bias in neoplastic cases (i.e., a tendency to recall the neoplastic cases but not the significant fraction of normal cases). As an example, from published data within a large reference lab [Ng et al], only 26% of peripheral blood cases show a B cell abnormality of which 18% of the total peripheral blood cases are CLL. Therefore, if CLL/SLL markers are included upfront, <3% of cases will require add-on studies (e.g., cases of B-ALL, CD10+ B-NHL, HCL).

Another example is the add-on strategy in working up new Acute Myeloid Leukemias (AML). While the overall diagnosis is typically trivial, lineage assignment by cytoplasmic antibodies, exclusion of rare erythroid and megakaryocytic AMLs may be required depending on the pathologists' level of comfort, the quality of specimens, and the ability to correlate clinical, morphologically and with other ancillary studies. While the additional markers in a 10-color space are relatively easier to include, in the absence of clear guidelines to what markers are 'necessary' to fully work up an AML by flow cytometry, this open question is best answered locally by the pathologists in cooperation with the technical staff given the significant additional work of these cases. Furthermore, as flow cytometry often over-estimates the blast percentage (due to erythroid lysis) compared with the gold-standard morphologic differential, decisions must be made regarding triggering levels for when AML add-on studies should be initiated (a 20% blast fraction by flow cytometry may be seen with 10% morphologic blasts)

In the work up on CD10+ B-LPD, some labs also use a cytoplasmic bcl-2 antibody to further define a population of light chain restricted germinal center B cells and help exclude a reactive process (Kussick et al). While not specific to 10 color flow cytometry, this assay may or may not be of clinical utility considering the availability of bcl-2 by immunohistochemistry, the quality of the specimen (e.g., excision vs FNA) or the degree of involvement by tumor.

Considerations in Panel Design

Approach

There are several approaches to consider for 10c panel design. One may simply merge existing 5c panels, develop entirely new panels and algorithms, or come up with a combination of the two. With merging of existing panels, redundant antibodies can be eliminated to make way for new ones, or redundancy may be leveraged to the panel's advantage. Redundancy may serve as a safety check, especially if a different clone or fluorophore is used. Among different 10c panels, it is advantageous to strategically place redundant markers on different panels for different purposes. For example, the use of CD34 may be used in a screening tube for bloods and bone marrows, and then again in myeloid and/or lymphoid leukemia tube(s). The use of a different clone and/or fluorophore, in this situation, provides reliable redundancy.

Another approach is to start 10c panel design from scratch. This approach is challenging and requires more work, but a thorough, global re-evaluation of the panels provides an opportunity for efficient optimization of panels to the laboratory's needs. To build from the ground up, it is essential to outline the laboratory's goals and needs, gather literature resources, and find updated diagnostic, prognostic, and therapeutic protocols for diseases of interest. The EuroFlow antibody panels are a great place to start. These are published, peer-reviewed consensus panels with testing algorithms that are evidence-based.

Map out how you envision an ideal workflow, with screening tubes, broad panels, or specific disease tubes. Screening tube design will differ from add-on tube design. What populations do you need to characterize in each tube? What markers do you need to carry over to add-on tubes to identify relevant populations downstream? Create a list of "must-have" antibodies that you cannot live without. Evaluating which markers are essential in certain tubes and why is critically important. This will be different for every lab.

Benefits

The increase in the number of colors detected in 10c brings an opportunity to design panels in such a way as to increase sensitivity for detecting abnormalities. This holds true especially with populations that have aberrant immunophenotypes, as multiple different lineages characterizing markers can be used to isolate populations. For example, having CD19 and CD20 in the same tube would catch a CD20-negative CLL, or a patient with a B-cell lymphoproliferative disorder that has been treated with anti-CD20 therapy. Similarly, a panel that includes multiple T-cell markers could isolate a T-cell lymphoproliferative disorder lacking a classic T-cell antigen.

The number of coloring options for different cell populations also increases with 10C flow cytometry. The ability to use multiple different visual colors for different cell populations in a sample provides an opportunity to reduce 2x2 plots. This can simplify evaluation tremendously.

General Rules

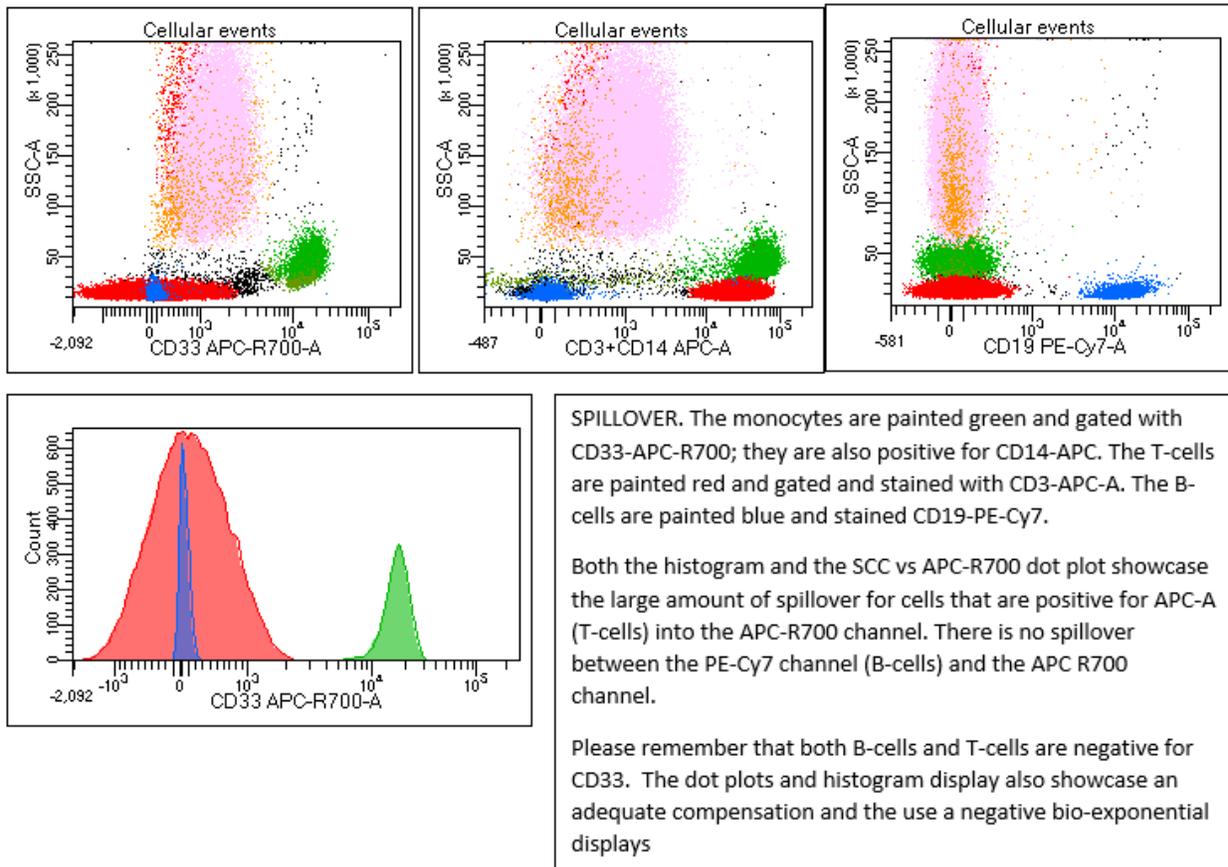
Full panel design steps with examples will be covered in a future module, but we will provide some general guidelines. In general, it is wise to:

- Pair bright fluorophores with weak antibodies and dim fluorophores with strong antibodies.
- Build in redundancy of markers as well as with populations.
- Pair fluorophores that tend to receive lots of spillover (e.g., APC, PE) with strong population markers (e.g., CD138 or CD38 for plasma cells).
- Pair fluorophores that generate lots of spillover with mutually exclusive populations (e.g., CD19 APC-A700 spillover into CD3 APC does not present a diagnostic challenge, as CD3 and CD19 are not expressed on B and T cells respectively).

Panel validation considerations (focus on 5 color to 10 color validation)

Once you have designed your new 10 color panels, laboratory quality practices will require you to validate your new tubes. As an initial step you will need to optimize new titrations for your new antibodies to obtain the best optimal signal to noise ratio and also minimize the volume of antibody used. In order to facilitate the titration of the reagent and to obtain more constant results in your assay

afterwards it is also recommended that you aim to stain the same absolute number of cells per tube. A cell count should be obtained after initial processing of the samples and highly cellular samples should be diluted; of note this will not be needed for low cellular samples. For example, one may decide to adjust their patient samples (peripheral blood, bone marrow, and tissue cell suspensions) to a concentration of $\sim 7.5 \times 10^9/L$ as needed and use 100ul of this adjusted patient cell suspension to a constant volume of antibody reagent. Once you have established the titration and volume to use for each antibody, it is recommended to double check and compare these individual titrations to the full combination and ensure that you still have a similar and good signal to noise ratio with the complete antibody combination. These two steps of adequate titration of your antibodies and a fairly constant absolute number of cells stained will greatly improve the resolution of your assay and standardized the appearance of the results; they are significantly more important in 10 color assays than in 4 or 5 color assays where the background noise (due to nonspecific binding and fluorescence spillover) of the assay is significantly less.



FMO and Instrument Settings

Once you have established a backbone panel design with the selected combination of fluorochromes and antibodies for each channel it is recommended that a validation of all FL channels with an fluorescence minus one (FMO) methodology be done to detect both compensation issues (including cross laser compensation) and unexpected antibody interactions (see also Q&S modules 2 and 8 (for Beckman Coulter systems), 3 and 20 (for BD systems), and the following link for further discussion on compensation: http://www.cytometry.org/public/educational_presentations/Roederer.ppt). This FMO validation of your panel maybe initially time consuming and onerous. However, it is very valuable in

order to gain experience and recognize the shift in settings and the greater background noise and spillover present in 10 color assays. An assessment and review of where the negative and positive populations should be made during this FMO validation. Note that the instrument settings such as the photomultiplier voltage also should be adjusted to have an optimal signal to noise ratio (see Wood, B for a discussion of PMT walking) and that the voltage settings should not be adjusted to keep the negative population on a lower log decade.

The brighter background noise of the 10 color assays as well as the greater spillover seen between some channels can move some negative populations to values that are 2-3 log higher than expected in intensity. This effect is one of the main disadvantages of 10 color assays versus 5 color assays and can cause a decreased sensitivity of low-level antigens if care is not used to carefully design panels to avoid these spillover effects (See Feske et al. for a discussion of consensus panel designs). Adjusting the settings of your instrument to set the negative population to a lower log decade will simply further decrease the sensitivity of the detection of that antigen in that FL channel.

Almost every 10 color clinical flow cytometry will utilize the same filter/mirror configuration for all panels in order to minimize the need to significantly change instrument settings and compensation values between panels and the extra work needed to 'revalidate' the machine between panel setups is often prohibitive. This is different from 4 and 5 color assays where the fluorochrome panels design can often change by using or not using tandem dyes; and laboratories would need to have separate optimized instrument settings and compensation values for different fluorochrome design panels.

The paragraphs above describe some of the steps and issues involved in optimizing 10 color assays before doing a comparison of your new 10 color assay with your current 4 or 5 color assays. Nevertheless, a documented qualitative and quantitative validation between your new 10 color assay and previous 5 color assay still should be made particularly in comparing and selecting the results you already report in your predicate 5 color assays. A qualitative and quantitative comparison of known cellular populations should be made (T-cells, B-cell, etc.) although you may not routinely report them. Noted that this comparison can be difficult for some results and populations that were not originally included in your 5 color assays. That is, a new 10 color assay will often provide additional new results and information such as new populations not previously routinely identified. The use of normal samples and establishing a reference of how a normal and selected abnormal samples look between your 5 and 10 color assays is strongly recommended including all add-on panels even though normal samples are not typically screened through them. Finally, for new/novel markers in a 10c panel, the use of a predicate method such as immunohistochemistry may be considered to confirm antibody specificity and performance.

Cocktail Validation

Finally, the use of premade cocktails is significantly more efficient with 10 color assays large volume laboratory practices. However, the stability of these cocktails must be validated. A new freshly made cocktail will need to be compared with an "aged" older cocktail using the same sample and instrument and ensure similar results between a new and an older cocktail. Depending on the volume and workload of the laboratory cocktails could be validated for one to several weeks if the laboratory has both a documented process and strict quality controls of its cocktails. It is recommended when checking the stability of the cocktails that both a qualitative concordance and quantitative assessment of the fluorochrome performance using values such "Stain Index" and MFI values is done. The older cocktail

should provide similar results to the new cocktail using the same assay procedure in place with respect to patient sample volume, instrument settings and compensation values (see Q&S module 16).

In particular the cocktail validation should include an assessment of possible tandem breakdown which could result in significant compensation errors (i.e., tandem breakdown issues cannot be compensated away). A procedure to optimize the stability of the cocktail such as using dark glass amber containers as well in premade small single sample assays “micro tubes” should be considered. A documented quality control validation of a new cocktail lot versus a previously made cocktail lot to ensure that all the antibodies have been added is highly recommended and, in some countries, also a regulatory requirement.

Other opportunities during transition

The field of flow cytometry has advanced leaps and bounds since the introduction of 5 color cytometers in the clinical lab (such as the FC500) almost 20 years ago. When transitioning from a 5 color to 10 color platform, it may be a good opportunity to evaluate other facets of your lab including software, collecting additional parameters and new gating strategies, updates to staining controls, increasing the events collected, and re-evaluating how staff competency is evaluated in a more artifact rich environment.

Software - Analysis and Reporting

While in the past, analysis and gating may have been performed on the same acquisition software, offline software packages have since become available and allow for gating and analysis separate from the cytometer computer. While this may add costs for additional software licenses, this has significant workflow advantages such as allowing individuals off-site access to the data and allow for simultaneous collection of cases on an otherwise fallow cytometer. Likewise, report generation functionality should be evaluated as well. The two major paradigms are report integration within the analysis software (e.g., FCS express) versus separate reporting and analysis (e.g., LIS or middleware reporting tools & Kaluza). These paradigms trade convenience in seamless data transfer from gate to report versus high performance in real time gate manipulation (particularly of large MRD files) and report generation that is best suited to your workflow and LIS. For example, an integrated solution like FCS express allows reports to feature complicated calculations within the text based on gated values without a need for manual transcription of values (a frequent cause of laboratory errors). However, a split model like Kaluza allows for real time manipulation/gating of large files and one’s workflow may utilize integrated reporting with the morphologic findings (e.g., flow reporting within a bone marrow report) which obviates the advantage of an integrated solution. These decisions are important and have significant consequences regarding throughput, ease of remote work (especially in the post-COVID19 world), trainee education, and staffing resources.

Gating Strategies in a 10 color world

In 5 color panels, inclusion of CD45 may or may not have been a luxury one was willing to trade off and instead forward scatter/side scatter (FS/SS) gating was the principal gating strategy in separating out leukocyte populations for phenotyping. In a 10 color panel, CD45 is considered a backbone (it is nearly unheard of to go without CD45 except in very specific and limited add-on assays) and so using a combination of FS/SS gating to seek out viable events (assuming you chose to not use a viability marker, DAPI, PI etc.) followed by CD45/SS gating can result in very clean gating of hopefully viable events without significant nonspecific staining. Likewise, additional non fluorescent parameters are often available on new 10 color instruments that may not have been available or in frequent use when 5 color clinical instruments were introduced. These include time parameter, forward scatter peak/height and forward scatter area/integral. These allow for time gating to exclude clogs and bubbles as well as singlet

gating to exclude double-events and in the modern era of clinical flow cytometry are considered indispensable quality management tools that can be had for “free”.

Likewise, differential coloring of populations was less needed in a 5 color space because of markedly the lower dimensionality (10 unique 2x2 plots). However, in the increased dimensionality of a 10 color space, backgating with complementary colors may make it easy to spot light chain restricted populations (for example blue for kappa+ B cells and red for lambda+ B cells) without having to review all possible unique 10 color combinations (i.e., 55 plots)

FMO vs Isotype controls

While not specific to 5 color to 10 color panels and transitions, updating controls where needed should be considered when updating panel designs. Classically, isotype controls were chosen to serve as a negative threshold but have since gone out of vogue in most laboratories. Internal negative controls or FMO (fluorescence-minus-one) controls are often a better (and cheaper) choice depending on what is being controlled for [Hulspas et al]. Isotype controls may still have some use as controls for cytoplasmic antibodies because of the potential of neoepitope formation and the “stickiness” of the cytoplasm but noted that some newer dyes may not work very well in FMO controls. For example, brilliant violet dyes (BV421) can show significant nonspecific binding of the dye rather than antibody due to the molecular structure of the dye.

Increased Event Collection

One of the major advantages of 10 color flow is the ability to run fewer tubes/panels which results in more available sample volume per tube and more average number of events per tube. While the intention of these L/L panels may be not for minimal residual disease (MRD) testing, in collecting large numbers of events, the same benefits and pitfalls will be evident. But like many benefits, this is not without costs - run times may increase and plots and samples may look ‘dirtier’ eliciting complaints from the medical staff as the increased number of events highlight heretofore unobserved small populations or small numbers of non-specific binding events. As most modern software allows for changes in the displayed dot resolution/size, it is recommended that rare event analysis be done with lower resolution or with rare event emphasis to allow spotting these small populations and characterizing their phenotypic aberrancies. In addition to the interpretive challenges that may arise, increased event collections will increase sensitivity for small, perhaps clinically insignificant populations such as monoclonal B cell lymphocytosis (MBL) and/or small populations of light chain skewed or restricted B cells which may be reactive rather than neoplastic. Among T cell populations, this issue is particularly troublesome since small reactive and clonal T cell populations are not infrequent, and which are not the harbingers of neoplasm. Without proper training and an understanding of these populations, there may be an increased diagnosis of suspicious populations leading to increased clinical follow-up without clear patient benefit.

Staff competency

The artifacts and increased complexity of compensation errors seen in 10 color flow cytometry raise the need for increased staff training. How best to accomplish this will depend on the workflow and experience of the technologists and pathologists. Continuing education programs such as ASCP specialist certification in cytometry (SCYM (ASCP) <https://www.ascp.org/content/board-of-certification/get-credentialed>) may be of utility to establishing staff competency. Additionally, didactic lectures on artifacts and compensation by the medical directors and development staff explaining the pitfalls of the new assay(s) may be helpful. Beyond the technical aspects of switching to new assays and updating the standard operating procedures (SOP) in the lab, educating technologists regarding the rationale for: 1.) switching over to 10 color platforms, 2.) specific design decisions in the panels, 3.) new

paradigms underlying the add-on strategies is often appreciated and gives a sense of ownership to the technologist regarding the performance of these assays. For a further review of the topic staff education and training we refer you to the excellent consensus statement by Greig et al.

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