ADVANTAGES AND LIMITATIONS OF IMMUNOPHENOTYPING AND LYMPHOCYTE PROLIFERATION ASSAY IN CLINICAL FLOW CYTOMETRY IN THE FIELD OF PRIMARY IMMUNODEFICIENCY DISEASES

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Abstract

Flow cytometer, a machine that utilizes the usage of light signals, has become one of the important machines or components in the studies of specific cell populations, cell surfaces, or even in detecting intracellular proteins. Due to its multiplex functionalities, while at the same time being very sensitive and specific, flow cytometer, or the technique of flow cytometry, has become a favourable and valuable tool for immunologists and clinicians for initial diagnosis of primary immunodeficiency diseases (PID), especially in studying cellular functional characteristics linked to PID. This paper will review key immunology tests of clinical flow cytometry, specifically in immunophenotyping, T cells classification and lymphocyte proliferation assays for their advantages and limitations in the field of PID. Importance of validation for the assays will be discussed. It is expected that development of validated immunology tests in clinical flow cytometry for PID diagnosis ensure standardized protocols, improve troubleshooting and disease interpretation, and improve the quality in patients' diagnosis and management.

Keywords: Flow cytometry, Primary immunodeficiency diseases, T cells, Immunology tests, Immunophenotyping

Introduction

Primary immunodeficiency diseases (PID) is an umbrella term for genetic defect diseases involving the immune system. Also known as Inborn Errors of Immunity (IEI), suspected PID patients are those who typically manifest susceptibility against infectious diseases or opportunistic pathogens, and those who suffer from autoinflammatory diseases and/or autoimmunity. There are also reported cases of patients who succumbed to cancers due to PID (1). Genetic mutations in PID can cause loss of one or multiple protein expressions and changes in functionality of encoded protein. In a classic snowball effect, mutation of the monogenic germlines resulted a wide array of cellular defects. Examples are poor performances of important proteins linked to the functionality of immune cells or defected cells with critical roles in homeostasis and responses to external or internal stimuli. These abnormalities hence resulted an aberrant immunity of patients. Now, 485 PID with 55 newly discovered novel gene defects have been identified and characterized into 10 different subgroups, as per International Union of Immunological Societies (1).

Previously known to be rare, ongoing discoveries and studies have shown that the prevalence of PID is much common, reported to be as high as 1 in 1200 (2). PID in Asia have been described to be varied. In addition, manifestation of distinctive disease pathologies and morphologies have been reported (2). Delayed or misdiagnosis is common in PID. In Malaysia, gaps in terms of knowledge, translational diagnosis and management of PID have been identified (3). Data, awareness, and diagnostic facilities are some of key components in managing PID at a national level. Challenges due to lacking in any of the key components may contribute to preventable suffering and deaths (4). For example, SCID patients will not survive beyond the first year of life if patients failed to receive allogeneic hematopoietic stem cell transplant (4).

Early diagnosis of a potential PID patient is important to initiate a proper patient's management. Further, improvement in early diagnosis will help to reduce the infection-associated morbidity and mortality rate. Genetic tests are the best tools for PID diagnosis (5). However, they are time-consuming, laborious, and expensive for patients that require immediate care, or patients that are coming from low to middle income families. Exome sequencing which is gaining attention for diagnosing PID is also hampered with limitations, specifically in terms of costs, labour intensity, and turn-around time (5). As such, initial screening of PID routinely involves the usage of flow cytometry, an equipment with a quick turn-around time, besides being more cost effective as compared to genetic tests. Flow cytometry technique is also quantitative, an important factor in diagnostic perspective. In addition, being relatively easier to conduct gives it an added advantage against other tedious laboratory techniques, making it widely available and are much in favour for scientists conducting PID screening tests (6). This review highlights, discuss and gives an insight about flow cytometry-based immunological tests and their crucial contribution in early diagnoses of PID patients.

Materials and Methods

This is a narrative review describing and discussing the fundamentals of clinical flowcytometry and the application in characterisation of the immunophenotyping, T cells classification and lymphocyte proliferation assays. We conducted a search in PubMed for published scientific articles in relation to keywords "clinical flowcytometry" and identified the relevant articles. We summarize and synthesize the findings and presented in this narrative review.

Clinical flowcytometry

Flow cytometry utilizes the usage of light beams and fluidic system (7). Via the fluidic system, cells of interest will move one by one, precise and uniform, through the region of liquid stream known as flow chamber. Focused light beams will then illuminate each cell passing through the flow chamber at a uniform rate. This interaction will produce scattered light signals that are picked up by two different light collection optics known as forward scatter (FCS) and side scatter (SSC). Obtained data will then be analysed by a specific flow cytometry software (7) (Figure 1). In flow cytometer, FCS is positioned to detect light scatters that are running through the laser pathway, and light diffractions will allow detection of cells with multiple different sizes. On the other hand, SSC is located at a ninety-degree angle away from the laser. Light scatters detected at SSC are due to light refracted from interaction of the beam and intracellular structures, and so, information obtained from SSC will be the reflection of cells granularity or complexity (7) (Figure 2). Typically, cells of interest are stained with organic dyes of heterocyclic structures known as fluorochrome, prior to analyses (8). Stained cells will be excited by light at specific wavelengths allowing measurement of the fluorescence at different filters. As each dye has their own specific excitation wavelength, an appropriate panel design will result in good assay with limited overlapping of emission spectra. Choosing a suitable pair of conjugated dyes, apart from considering available lasers for the analyses is important (8).

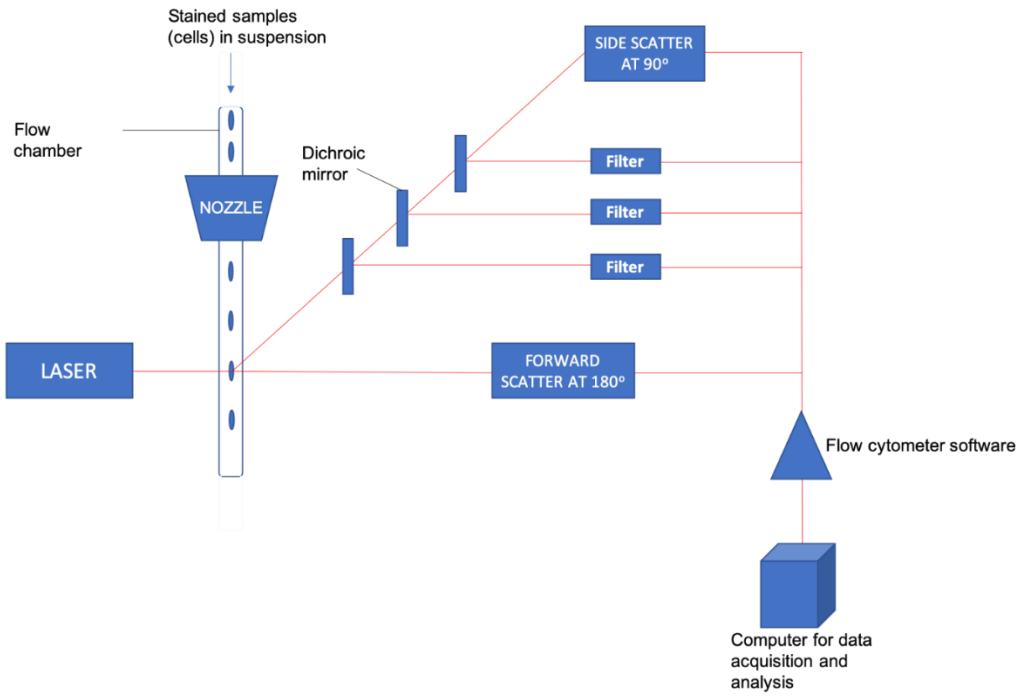

Figure 1: A simplified schematic diagram of flow cytometer

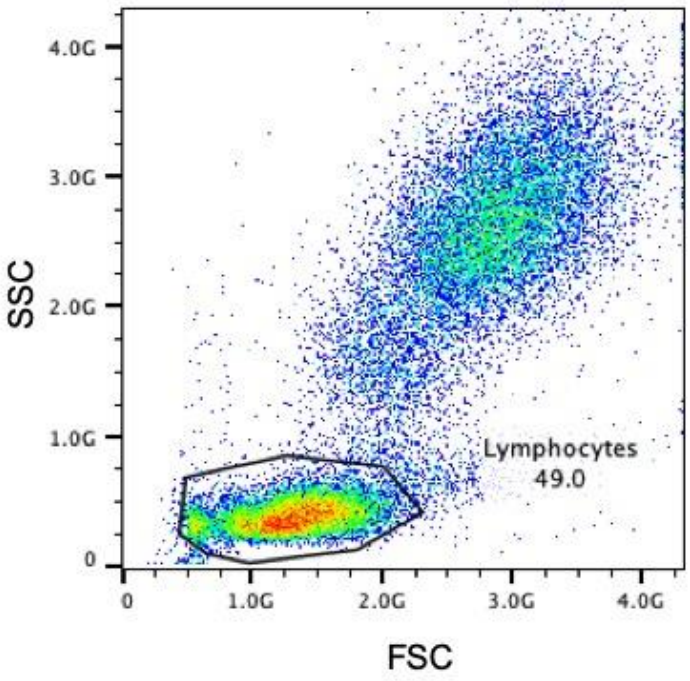

Figure 2: An example of cell populations analysed using FlowJo software. The cell populations are distinguishable by their cell sizes and granularity

In the studies of PID, flow cytometry is sensitive enough to evaluate immunophenotype, intracellular proteins and activation markers, as well as evaluation of cellular functions from specific cell populations. For example, immunophenotyping assay allow for the analysis of altered T and B cells subpopulation, in addition to alterations of NK cells, monocytes, and dendritic cells. Moreover, flow cytometry has an edge in evaluating expressions or alterations of proteins suspected to be linked to PID. Lymphocyte proliferation assay is one example of an assay that makes full use of flow cytometry multiplexity in evaluating T cell function (9). In this assay, mitogens (a well-known T-cells activation and proliferation stimulators), are used to study the functional ability of lymphocytes to proliferate upon stimulation. Example of mitogens are phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) (10).

Defects in lymphocyte populations or subpopulations, cell surface protein expression, or functional abnormalities in initial screening using flow cytometry will be critical in rapid patient's managements and subsequent genetic testing (11). Development and validation of these immunology tests, specifically in immunophenotyping and lymphocyte proliferation, ensures quality and reliability of these assays that contributes to disease interpretation and patients' management.

Immunophenotyping

Abnormalities in lymphocyte populations (T, B and NK cells) can be an early clue of PID. Different PID types will have different presentation in terms of lymphocyte subpopulations.

For example, there are multiple reported cases of severe combined immunodeficiencies (SCID) patients expressing defects in production of immune cells, namely T cells, B cells and/or NK cells, in contrast to patients suffering from agammaglobulinemia that are normally presented with an exclusive absent of B cells. PID cases with isolated low number of CD4, CD8, and NK cells have been reported and characterized as per IUIS (12). Although reduced number of T cells, B cells and/or NK cells can be the classic indication of PID patients, patients of common variable immunodeficiency diseases (CVID) introduce a complication in diagnosis as there are reported past cases with positive CVID patients presenting normal absolute count of T cells, B cells, and NK cells. However, in most cases related to CVID patients, although the absolute number of B cells is normal, patients demonstrated substantial abnormalities in B cells subpopulations and /or functions (12). Due to a wide spectrum of clinical and immunological presentation of PID, immunophenotyping is an indispensable tool in simultaneous analyses and screening of lymphocyte populations and subpopulations. This assay will therefore help to discriminate PID from one another. Moreover, the analyses will help clinicians to proceed with further genetic investigations and diagnosis.

Immunophenotyping utilizes fluorochrome-labelled antibodies for the detection of cells of interest. These conjugated antibodies have different binding sites allowing each labelled antibody to have specific targets of leukocyte surface antigens (13). Cells stained with these antibodies will run through the flow chamber of flow cytometer, illuminated by the laser beam. The final product, i.e., scattered signals, are then captured by the FCS, SSC and specific filters, ready to be analysed (13). There are many markers designed for specific cells of

interest. Table 1 summarizes cell markers that are normally used in PID diagnoses.

Table 1: Major cell markers in PID diagnoses

Cells of interest		**Phenotype**
Granulocyte		$CD45^+/CD15^+$
Monocyte		$CD45^+/CD14^+$
T cells		$CD45^+/CD3^+$
$CD4^+$ T cells		$CD4^+/CD8^-$
	Naïve $CD4^+$	$CD27^+/CD45RA^+/ CCR7^+$
	Central memory $CD4^+$	$CD27^+/CD45RA^-/ CCR7^+$
	Effector memory $CD4^+$	$CD27^-/CD45RA^-/ CCR7^-$
	Terminally differentiated (TEMRA) $CD4^+$	$CD27^-/CD45RA^+/ CCR7^-$
$CD8^+$ T cells		$CD4^-/CD8^+$
	Naïve $CD8^+$	$CD27^+/CD45RA^+/ CCR7^+$
	Central memory $CD8^+$	$CD27^+/CD45RA^-/ CCR7^+$
	Effector memory $CD8^+$	$CD27^-/CD45RA^-/ CCR7^-$
	Terminally differentiated (TEMRA) $CD8^+$	$CD27^-/CD45RA^+/ CCR7^-$
B cells		$CD45^+/ CD3^-/CD19^+/CD16+CD56^-$
Pre germinal center B cells		$CD27^-/IgM^+/ IgD^+$
Unswitched memory B cells		$CD27^+/IgM^+/ IgD^{+/-}$
Switched memory B cells		$CD27^+/IgM^-/ IgD^-$
NK cells		$CD3^-/CD19^-/CD16+CD56^+$

Choosing fluorochrome-antibody pairing is also important since fluorochromes project different signal intensities upon excitation. In studying cell populations with multiple markers of interests, dim fluorochromes are normally paired with cell populations that are high in number, and vice versa. Doing so helps to prevent signal bleedings and overlaps. Commonly used fluorochromes and their brightness properties are listed in Table 2.

Another issue when running multiple markers is signal spill-over. As fluorochromes have wide emission spectrums, upon excitation, multiple lasers can accidently excite one fluorochrome, resulting in spilling its signal unto other channels meant for other markers. To correct these emission spectra, single stained cells are introduced as controls and allow the flow cytometer to recognise specific light signal, i.e., fluorochrome, for its own specific detector.

Table 2: Commonly used fluorochromes. When designing a panel, note that relationship between cell population and fluorochromes signal intensity is inversely proportional

Fluorochrome	**Brightness intensity**
BD Horizon BV421	Brightest
BD Horizon BV650	Brightest
BD Horizon BV711	Brightest
BD Horizon BB515	Brightest
BD Horizon BB700	Brightest
BD Horizon PE-CF594	Brightest
PE-Cy5	Brightest
BD Horizon BUV563	Bright
BD Horizon BUV661	Bright

BD Horizon BUV737	Bright
BD Horizon BV480	Bright
BD Horizon BV605	Bright
BD Horizon BV786	Bright
PE-Cy7	Bright
PE	Bright
APC	Bright
Alexa Fluor 647	Bright
BD Horizon APC-R700	Bright
BD Horizon BUV395	Moderate
BD Horizon BUV496	Moderate
BD Horizon BV510	Moderate
FITC	Moderate
Alexa Fluor 488	Moderate
PerCP-Cy5.5	Moderate
BD Horizon BUV805	Dim
BD Horizon V450	Dim
BD Horizon V500	Dim
PerCP	Dim
Alexa Fluor 700	Dim
APC-H7	Dim
APC-Cy7	Dim

T cell subset classification

T cell subset classification is a further specific immunophenotyping of T cells subpopulations. In fact, in decision-making situations related to suspected PID cases, getting a good analysis of the T cells subsets are pivotal and will help to navigate the direction of treatments. This is because, the subset analysis will provide good information regarding the condition of atypical T cells in the lymphoid system; namely reduced cells count or abnormal maturation (14). Cluster of differentiation (CD) is the term, or nomenclature that are being used for classification of unique surface antigens. This nomenclature helps to characterize different immune cells in a systematic manner. T cells can be categorized into two main groups which are $CD4^+$ that represent T helper cells and $CD8^+$ that signify cytotoxic T cells. From these two groups, there are subsets of T cells that can be discriminated by analysing the expression of CD45RA/CD27/CCR7 and CD45RA/CCR7 in the group of CD4 and CD8 respectively (15). In $CD4^+$ group, cells with $CD45RA^+CD27^+CCR7^+$ indicate naïve T cells while $CD45RA^-CD27^+CCR7^+$ cells are central memory T cells. $CD45RA^+CD27^-CCR7^-$ suggested terminally differentiated T cells while $CD45RA^-CD27^-CCR7^-$ represent effector memory T cells (Figure 3). In $CD8^+$ groups, subsets of T cells are naïve T cells ($CD45RA^+CCR7^+$), central memory T cells ($CD45RA^-CCR7^+$), terminally differentiated T cells ($CD45RA^+$, $CCR7^-$) and effector memory T cells ($CD45RA^-$, $CCR7^-$) (15, 16). These subgroup of T cells are critical in providing initial ideas on the functionality of T cells of patients. $CD4^+$ naïve cells, more importantly, can also become an indicative marker of recent thymic emigrant (RTE) cells. RTE are newborn T cells that just completed their development and egress to join and maintain the T cells population (17). As such, reduced absolute count of $CD4^+$ naïve cells indicate low number of RTEs, and consequently gives an idea about the functionality of the thymus. Naïve T cells immunophenotyping may not be as accurate as T cell receptor excision circles (TREC) screening, an assay quantifying TREC that reflects T cells development. However, naïve T cells being as indicative marker of RTEs can give physicians an initial assessment regarding thymus functionality of patients.

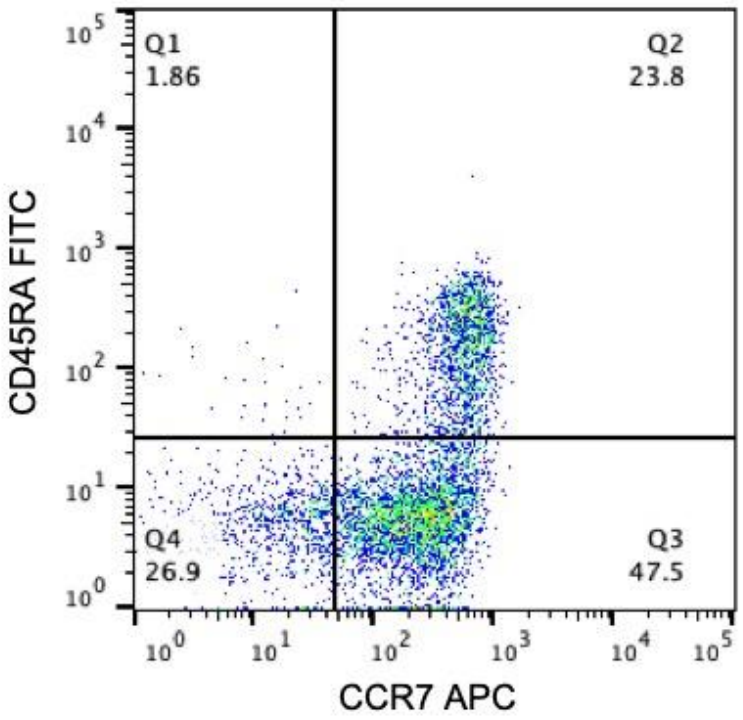

Figure 3: Example of T cells classification of $CD4^+$. T cells are labelled with CD4, CD45RA and CCR7. The resulting combination markers are terminally differentiated cells (Q1), naïve cells (Q2), central memory cells (Q3) and effector memory cells (Q4)

T cells lymphocyte proliferation assays

Lymphocyte proliferation assay is another laboratory technique that utilizes the usage of flow cytometry to monitor any impaired T cells functionality of an individual. In addition, since T cells, B cells, and NK cells cross-talk with one another, monitoring functionality of T cells is critical as the outcome may imply the functionality of B and NK cells. It has long been known that mitogens such as PHA, Con A and PMW are capable to induce T cells to proliferate, regardless of the cellular behaviour of the T cells. T cells may also be stimulated and proliferated via introduction of antibodies against T cells receptors CD3 and CD28. Any observed abnormal T cells proliferations are indicative of aberrant T cells function (18), and likewise, may contribute to abnormal B cells and/or NK cells. It has also been reported that cellular proliferation analysis will be helpful in determining the presence and frequency of antigen specific T cells. This is because information that are obtained from this analysis will provide insight on how the cells respond and modulate after being exposed to specific antigens or therapies.

In this assay, isolated peripheral blood mononuclear cells (PBMC) are stimulated in vitro with mitogens or antigens, cultured for minimum 72 hours before being analysed using flow cytometer. The non-specific binding of mitogens to the carbohydrates of T-cell Receptor (TCR) or CD3 will trigger the intracellular signalling of T cells. The signalling cascade will then induce T cells activation and ultimately T cells proliferation. On the other contrary, CD3/CD28 antibodies are co-stimulator that directly interact with TCR and induce T cells proliferation.

While CD3/CD28 antibodies are specific to T cells, some mitogens will also activate and instigate B cells proliferation. Known mitogen that stimulate B cells is PMW, although the mechanism is still unclear (19). NK cells on the other hand, are not affected in this in-vitro stimulation. Lymphocytes are also labelled with $CD3^+$ marker to prevent false gating of proliferated B cells and NK cells in the lymphocyte population.

In-vitro lymphocyte proliferation is usually monitored using two methods: a dye-based proliferation assay and ^{3}H-thymidine-based assay (20). ^{3}H-thymidine during DNA synthesis remain the golden standard for measuring T cell proliferation (20). Via this method, cells are incubated with ^{3}H-thymidine for few hours to overnight, allowing integrations of radioactive labelled thymidine into the nascent DNA of proliferating cells. A simple and easy method with high throughput (20), ^{3}H-thymidine method has several major drawbacks including being cumbersome due to the usage of radioactivity and no discrimination of specific cell subpopulations that responded upon stimulation. Moreover, ^{3}H-thymidine method is also unable to provide information on factors that contribute to activation-induced cell death, in addition to being unable to discriminate factors behind the decrease of lymphocyte proliferation for results interpretation.

Dye-based proliferation assay was developed over the past decades, and currently there are a variety of fluorescent dyes with different excitation signals readily available to monitor T cells proliferation (20). Example of a common fluorescent dye is carboxyfluorescein diacetate succinimidyl (CFSE). In this method, the fluorescent dye will diffuse into the cells and bind covalently to the amino

group of intracellular proteins. When cell division occurs, CFSE will be divided equally among the daughter cells, in which the process will continue until eight daughter cells generations. This phenomenon resulted fluorescent intensity that is reduced by half for each subsequent daughter cells populations (20, 21). Analysed by the flow cytometer, distinct peaks can be observed. Peak with the highest fluorescence intensity indicate parent cells population while peak with the lowest mean of fluorescence intensity signify the most recent daughter cells population (Figure 4). The number of peaks will give an insight on the number of cell divisions that have occurred (20). In addition, the proliferated cells can be labelled with specific markers, allowing phenotypical characterization and analysis via flow cytometry. Known for its stable incorporation of the dye, dye-based proliferation assay, for example CFSE, does have several drawbacks including the need for *in vitro* labelling prior to cell division. On top of that, CFSE is only able to monitor maximum eight cell divisions, and more importantly, cells lost may occur due to asymmetric cell division and cellular toxicity due to the dye (22).

Click-iT-EdU, a method that uses a nonradioactive DNA approach, can be used to monitor T cells proliferation, and has shown results that are comparable to ^{3}H-thymidine assay (22, 23). In this method, cells undergoing cellular division are fixed while detergent permeabilization allows 5-Ethynyl-2'-deoxyuridine (EdU), a thymidine analogue containing an alkyne, to be incorporated into the cells. EdU then reacts with fluorescent azide dye via a copper-catalysed reaction (click reaction) to form a stable covalent bond. As the reagent is small, the click reaction happens without harsh cell treatment. Click-iT-EdU can multiplex with most standard antibody conjugates, fluorescent proteins, cell cycle dyes, and cell surface and intracellular markers; giving it a wider range of cells analyses. This advantage, on top of being less harmful (no radioactivity and cellular toxicity) makes this assay a favourable approach as compared to its counterparts; i.e. dye-based proliferation assay and ^{3}H-thymidine-based assay (20, 22). Click-iT-EdU is also able to detect specific S phase of proliferation, in addition to being able to monitor cells divisions higher than eight daughter populations, two data interpretations unachievable by the other two aforementioned assays (Figure 5). It is important to have a good data comparing these assays to determine the best lymphocyte proliferation assay in terms of efficiency, results output, and consistency.

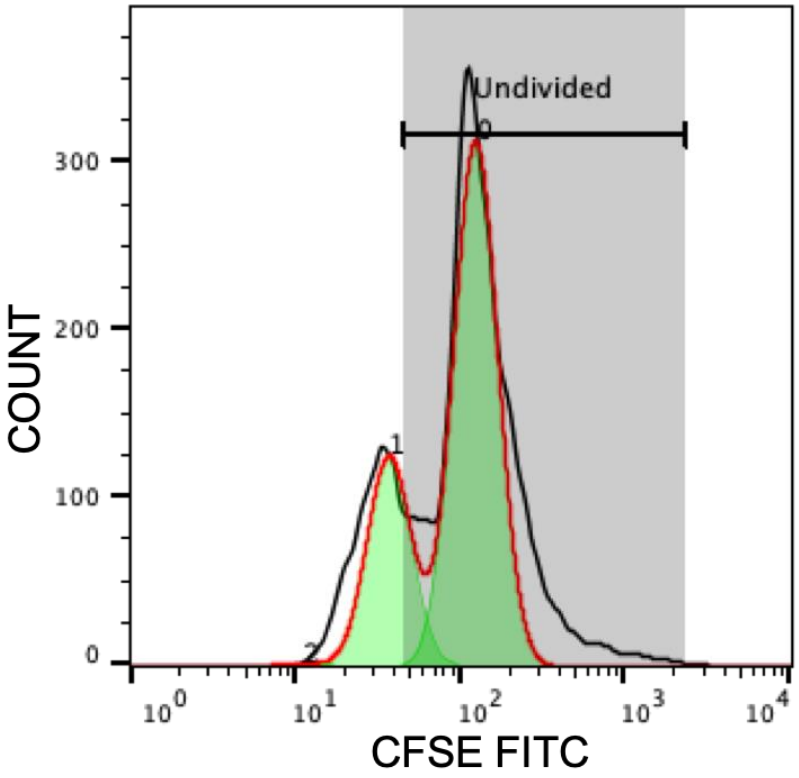

Figure 4: Example of lymphocyte proliferation assay analysed using FlowJo software. The left peak indicated daughter generation. There may be multiple peaks indicating different daughter cell generations, with the most left being the most recent. Peak at the furthest right indicate parent cells

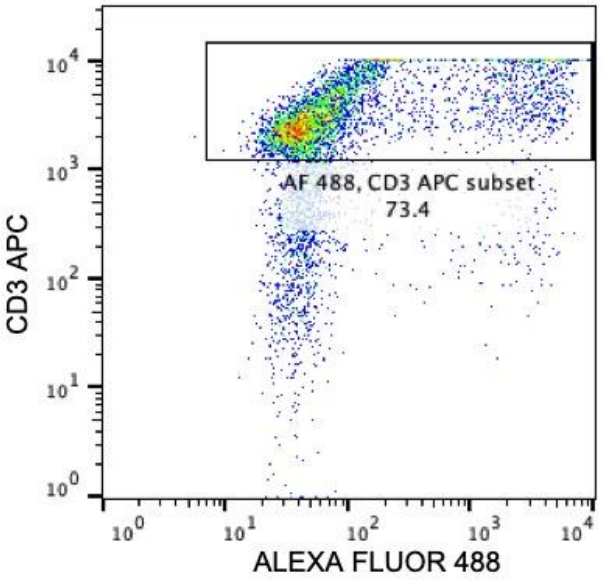

Figure 5: S phase proliferation observed using Click-iT Edu Assay

Assays limitations

In summation, immunophenotyping of lymphocytes, and classification of lymphocytes subsets allow an overview of general lymphocyte populations, determine specific abnormalities of lymphocyte subsets, and investigate if abnormalities are due to PID or other factors e.g. infections. Restoration of lymphocyte functionality after stem cell transplant may be observed via immunophenotyping. Immunophenotyping however does has its limitations. Being an assay that directly access and quantify surface markers only, this assay is not able to detect cellular dysregulations, which include inability to discriminate defects due to modified cytokine/chemokine environment and inability to have a pin-point diagnosis of lymphocyte functionalities. Example of lymphocyte functionalities are oxidative burst, NK cells cytotoxicity and T cells proliferative function. As such, it is recommended that follow up tests using a more sensitive tools and protocols are conducted upon immunophenotyping investigation. For example, in the case of suspected cytokine/chemokine defects, subsequent tests of serological cytokine and chemokine measurements using cytometric bead array (CBA) or enzyme-linked immunosorbent assay (ELISA) is recommended (12).

Lymphocyte proliferation assay is typically monitored via dye-based proliferation assay and ^{3}H-thymidine-based assay. Having data access into the T cells proliferation is important as cellular functionality information is inaccessible solely through immunophenotyping. There have been PID cases with normal T cells absolute count but impacted T cells function. Unfortunately, the decades old assays have their limitations which may impacted the analysis of results. Labelling by ^{3}H-thymidine into the DNA is toxic to the user due to its high level of radioactivity. Dye based proliferation assay on the other hand has limited information regarding daughter cells, other than being toxic to the cells. A method that can overcome these limitations is thus necessary. Click-It EdU, which is a gentle assay may be a good alternative to monitor T cells lymphocyte proliferation.

ISO 15189 – Medical laboratories

When providing diagnostic tests, quality is key. It is thus essential that laboratory running these tests follow ISO 15189, First published in 2003, ISO 15189 is a regulation dedicated to clinical laboratories, with listed guideline that ideally fits any laboratories conditions. Under this international standardized regulatory, a medical laboratory is highly scrutinized to conform to ISO 15189. In turn, the laboratory will develop a harmonized good quality management system (QMS), results are highly reproducible globally and therefore, build its competence in producing consistently reliable tests (24). For accuracy and reproducibility testing, external laboratory comparison should be conducted, in which the external laboratory must also follow ISO 15189 regulations. Maximum variation coefficients as per ISO 15189 criteria is 20% (12). However, it is well known that there are many difficulties in integrating flow cytometry to strictly follow the guideline. As flow cytometer is multiplex, it can be used in multiple disciplinary that has their own rules and regulations. As such, depending on the department that handles the flow cytometer, the machine may be subjected to multiple body accreditations, for example Good Clinical Laboratory Practice (GCLP) or Good Manufacturing Practice (GMP). To overcome the confusion, peer review guidelines and professional courses known as Clinical and Laboratory Standards Institute (CLSI) has been developed specifically for cytometric laboratories (24). Nevertheless, lack of finance to maintain the accreditation, in addition to having no specific or formal training to produce certified cytometrists are some challenges in standardizing flow cytometry globally. Other than that, diagnostics panels are also limited, meaning many flow cytometry tests are

developed and scientifically validated without gold standards. In tackling these shortcomings, several guidelines have been established, albeit with challenges, including instruments must be calibrated and the instrument's general performances are standardized. For calibration, usage of synthetic particles such as beads or microbeads is the standard practice (24).

Due to the many difficulties, integrating flow cytometry into ISO 15189, and to be a routine standardize test certainly is a tall order. As such, its standardization process is lacking as compared to other laboratory instruments. Yet, critical points listed in the guideline can still be adopted which in turn allow scientists to at least control the replication and reproducibility of results.

Conclusion

Clinical flow cytometry is a key component for a quick initial screening of potential PID patients. Laboratory techniques and assays, i.e., immunophenotyping, T cell subsets classification and lymphocyte proliferation are useful in providing critical data for any underlying immunological defects. However, due to complexity of PID cases and limitations and drawbacks of the assays, subsequent tests that tackle the limitations are highly recommended. Although Malaysia has yet to implement SCID screening, utilizing identification of T cell subsets specifically $CD4^+$ naïve T cells is imperative for initial screening of potential PID patients.

In addition, in lymphocyte proliferation assay, an improvement in detection will contribute have a much accurate diagnosis of patients' T cells proliferation. Even though ^{3}H-thymidine-based assay is the gold standard, it comes with a huge baggage of drawbacks. The introduction of Click-iT EdU may improve the precision and accuracy in diagnosis of T cells proliferative function.

Quantitative diagnoses, or even qualitative, are constantly loomed with the possibility of producing false positive or false negative results. It is imperative that the guideline introduced by the International Standard ISO 15189 - Medical Laboratories is followed. There are many challenges in adopting this guideline, however, the advantages outweigh the disadvantages. ISO 15189 guidelines will also ensure the medical laboratories are competent, able to produce quality diagnostic results, and consequently increase public confidence towards the said laboratories (12). All these accumulated data obtained will help clinicians in decision-making, especially for time-sensitive cases, hence improving the quality of patients' diagnosis and management.

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Competing Interests

The authors declared no conflict of interest.

Ethical Clearance

Ethical clearance was not obtained as this is a narrative review.

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