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HAEMATOPATHOLOGY

Polychromatic flow cytometry in the clinical laboratory

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Summary

Technological advances in flow cytometry include increasingly sophisticated instruments and an expanding range of fluorochromes. These advances are making it possible to detect an increasing number of markers on a single cell. The term polychromatic flow cytometry applies to such systems that detect five or more markers simultaneously. This review provides an overview of the current and future impact of polychromatic flow cytometry in the clinical laboratory. The use of multiple markers has several advantages in the diagnosis and monitoring of haematological malignancies. Cell populations can be analysed more comprehensively and efficiently, and abnormal populations can be distinguished more readily when normal counterparts are present. Polychromatic flow cytometry is particularly useful in the evaluation of plasma cells, and the role of flow cytometry in the assessment of plasma cell disorders is reviewed in depth. There is improved sensitivity in the assessment of small populations, which is critical in the evaluation of minimal residual disease. Flow cytometry can also play a role in assessment of circulating tumour cells in carcinoma. Introduction of polychromatic flow cytometry is a complex process with many challenges including design of antibody panels and instrument compensation. Developments in data analysis are required to realise the full benefits of the other technical advances. Standardisation of protocols may reduce inter-laboratory variation. While the complexity of polychromatic flow cytometry creates challenges, it has substantial potential to improve clinical analysis.

Abbreviations: ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CLL, chronic lymphocytic leukaemia; CTC, circulating tumour cells; LAP, leukaemia-associated phenotype; MRD, minimal residual disease; PFC, polychromatic flow cytometry.

Key words: Flow cytometry, fluorochromes, informatics, leukaemia, lymphoma, multiple myeloma, quality control, standardisation.

INTRODUCTION

Flow cytometry has a number of features that make it a valuable technique in diagnostic pathology. The strengths of flow cytometry include rapidity of analysis, ability to detect and enumerate small abnormal populations in the presence of normal cells, and capacity to assess several markers simultaneously on the one cell. Flow cytometry has an important role in the assessment of haematological malignancies, and this role has been reviewed in *Pathology,*[1,2] in other journals,[3,4] and in books.[5,6] The CD (cluster of differentiation) system introduced by the Human Leukocyte Differentiation Antigen (HLDA) Workshops has been invaluable in characterising markers for assessment by flow cytometry.[7] The rate of introduction of new CD markers into the clinical laboratory has slowed since the 1990s (Fig. 1). However, clinical laboratories have achieved increasing success in the use of well-established markers to identify small abnormal populations. This has been particularly important in the assessment of minimal residual disease. Other applications of clinical cytometry include analysis of lymphocyte subsets by flow cytometry, which was established in the 1980s and remains a key contribution of clinical cytometry, especially in immunodeficiency disorders such as AIDS.[8] Clinical cytometry also assesses haematopoietic stem cells by CD34 enumeration, and detects abnormal clones in paroxysmal nocturnal haemoglobinuria (PNH). Analysis of platelets and erythrocytes are further potential clinical applications of flow cytometry. While clinical cytometry has focused on disorders of the haematological and immunological systems, in blood samples it is also able to assess tumour cells from carcinomas.

This review focuses on some of the impacts of recent technical developments on the clinical laboratory. In particular, the review covers the role in the clinical laboratory of simultaneous assessment of more than four fluorochromes, often termed 'colours', on the one cell, a process known as multiparametric or polychromatic flow cytometry (PFC).[9] Developments in flow cytometry instrumentation and in fluorochromes are enabling the detection of ever increasing numbers of markers. It is now possible to evaluate 18–20 markers simultaneously on a single cell.[10,11] Research rather than clinical flow cytometry is driving the cutting edge of these developments. This point is illustrated in Fig. 2, which charts the history of research and clinical cytometers in one academic hospital and its associated research institutes and centres. In both research and clinical cytometry laboratories, there has been a steady increase in the capacity to detect simultaneous markers, but at any time the research facilities have generally been able to detect more markers than the clinical laboratory. Nevertheless, in recent years there has been a progressive introduction into clinical cytometry of instruments that perform PFC on 5–10 simultaneous markers. This review evaluates the role of PFC in the clinical laboratory, and outlines the challenges involved in establishing a quality service.

CONTRIBUTION OF POLYCHROMATIC FLOW CYTOMETRY TO THE CLINICAL LABORATORY

Examples in the diagnosis of haematological malignancies

Immunophenotyping has a key role in the diagnosis of haematological malignancies, and its place is recognised by the

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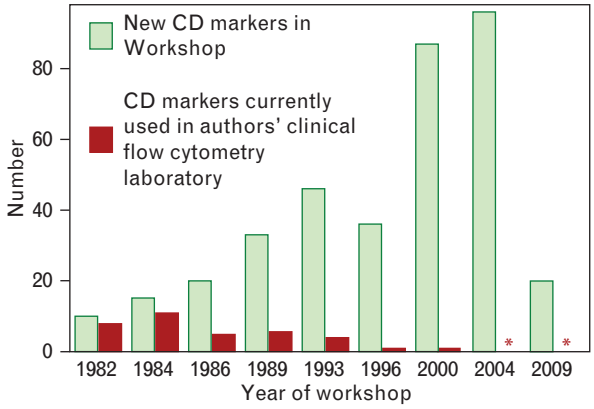

Fig. 1 CD markers used in clinical cytometry. The graph shows the number of new CD markers assigned at each of the nine HLDA Workshops, and the number of markers from each of these Workshops currently used in the authors' clinical cytometry laboratory at St Vincent's Hospital Sydney. *, no markers from the 2004 or 2009 Workshops are currently used in the authors' laboratory.

current World Health Organization classification.[12] Introduction of PFC has several benefits, including more comprehensive assessment of populations, detection of smaller subsets within large populations, and more information on paucicellular samples. However, in the diagnosis of haematological malignancies, there are few formal comparisons of PFC with earlier systems using four or fewer colours. Therefore, this review presents several examples to illustrate the power of PFC in diagnostic samples.

B cell disorders

A major role of PFC is in the analysis of complex populations. The availability of multiple markers enables populations to be separated and analysed independently. An example is shown in Fig. 3, where four-colour and eight-colour flow cytometry was performed on a lymph node fine needle aspirate (FNA). The four-colour analysis used antibodies to CD45 (for lymphocyte gating[13]), CD20, κ and λ, and the κ and λ analysis is displayed in Fig. 3A. In a separate tube, a substantial proportion of B cells was found to be CD10$^+$. Determination of the nature of these B cells is difficult with four-colour cytometry. However when the same sample was subjected to eight-colour analysis, the situation was easily resolved. The lymphocyte gate was determined by side scatter and CD45 [fluorochrome (FL)1]; cells with both

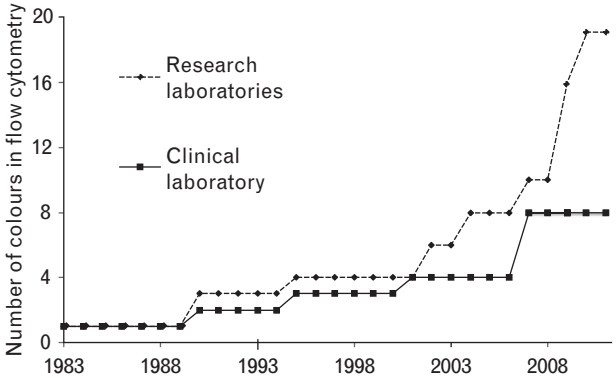

Fig. 2 Colour capabilities of research and clinical flow cytometry. Data are from St Vincent's Hospital Sydney and its affiliated centres and institutes. The graph shows the highest number of colours that could be simultaneously determined by flow cytometry in the research laboratories and in the clinical laboratory each year from 1983.

CD19 (FL2) and CD20 (FL3) are B cells, and are distinguished from T cells, which express CD3 (FL4). Clonality of B cells is assessed by expression of κ (FL5) and λ (FL6); this panel also includes two other markers useful in classifying B cells, CD10 (FL7) and CD22 (FL8).

In this case, the CD10$^+$ cells were essentially the same population as the bright CD20$^+$ cells (Fig. 3B,C). While the bright CD20$^+$ cells (essentially the CD10$^+$ B cells) were dimmer for κ and λ than the other B cells, there was no evidence of a monoclonal population (Fig. 3D). The presence of polyclonal B cells with CD10 and bright CD20 is consistent with reactive follicular hyperplasia.[14] PFC gives more confidence with reporting this type of sample than is possible with a four-colour system. In particular, in cases such as this, the pathologist can provide more reassurance that there is no monoclonal population.

PFC opens up the possibility of confidently identifying small populations and easily obtaining an extensive phenotype on them. A key strategy is to use the same three to four markers as 'backbone' markers in more than one tube. In the case of abnormal B cells, one of the tubes will also contain κ and λ, and this tube will provide the most powerful opportunity to identify a monoclonal population. In subsequent tubes, backbone markers are then used to identify the abnormal cells, and the phenotype is extended by further markers. In the case of B cells, a useful backbone is CD45 for lymphocyte gating and CD19 and CD20 for B cell delineation. The power of this approach in dealing with a small abnormal population in peripheral blood is shown in Fig. 4. The markers in the first tube were the same as in the previous example. A second tube contained the CD45, CD19 and CD20 backbone, and in addition the markers CD5, CD11c, CD23, CD38 and FMC7.

In the initial analysis, the abnormal cells were completely obscured by normal polyclonal B cells, and the initial κ and λ analysis was unremarkable, with κ$^+$ B cells making up 14% of lymphocytes and λ$^+$ B cells 8% (Fig. 4A). However, when the cells with the brightest CD19 and CD20 staining were selected (Fig. 4B), comprising 7% of lymphocytes, they proved to be essentially all κ$^+$ (Fig. 4C). PFC enabled other B cell markers to be assessed in this tube, and the abnormal cells were CD10$^-$ and bright CD22$^+$ (Fig. 4D). In the second tube, after CD45 was used to select the lymphocyte gate, CD19 and CD20 were again used to select the abnormal cells as in Fig. 4B. The phenotype of the abnormal population was then determined as CD5$^-$, CD11c$^-$, dim CD23$^+$ and FMC7$^+$ (Fig. 4E,F), and CD38$^+$ (data not shown). This approach would be very cumbersome without PFC. For example, in four-colour analysis, once three colours are allocated to the CD45, CD19 and CD20 backbone, then only one additional marker could be determined in each tube, and eight tubes would be needed to obtain the information provided by the two eight-colour tubes in this example. The examples in Fig. 3 and 4 demonstrate that the combination of CD19 and CD20 can be a very effective way of identifying B cell subpopulations. These subpopulations can then be efficiently assessed by PFC to obtain an extensive phenotype. In these cases, PFC improved the quality of the reports by determining the presence (in Fig. 4) or the absence (in Fig. 3) of monoclonal B cell populations in samples containing normal B cells.

T cell disorders

Flow cytometry can be used to assess clonality in T cell disorders by determining the expression of Vβ domains of the T cell receptor (TcR).[15,16] The principle is analogous to the

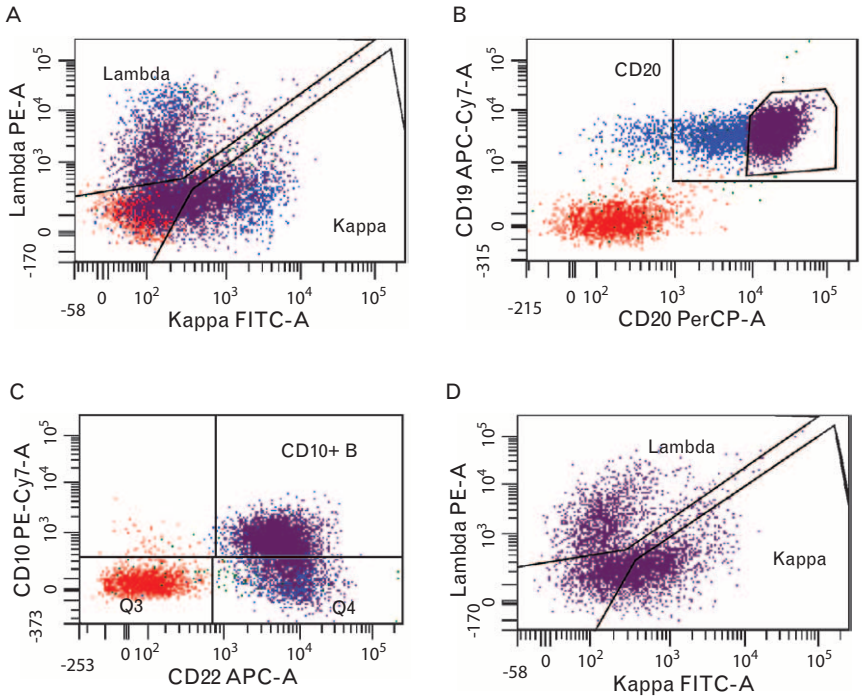

Fig. 3 Four-colour versus eight-colour analysis in a lymph node FNA. Lymphocytes were gated on CD45 and side scatter. Bright CD20$^+$ cells are displayed purple, moderate CD20$^+$ cells are blue, and CD20$^-$ cells are red. Panel A is from a four-colour analysis and Panels B–D from an eight-colour analysis. Panels A–C show all lymphocytes; Panel D only shows the bright CD20$^+$ cells. Analysis was performed on a FACSCanto II flow cytometer with a minimum of 10 000 lymphocyte events in each tube.

assessment of κ and λ expression to investigate B cell clonality. In practice, however, Vβ analysis is more complicated because instead of only two types of light chains on B cells, there are dozens of different Vβ specificities on T cells. This makes analysis expensive and labour intensive. Another disadvantage is that antibodies are only available to account for about 70% of T cells, and a similar percentage of cases of surface TcR α/β$^+$ T cell neoplasia.[17] Because of these limitations, Vβ analysis by flow cytometry is not widely employed.[3] However, there are advantages of a flow cytometric approach to assess T cell clonality, compared with molecular determination of TCR rearrangement. Flow cytometry is rapid, absolute numbers of

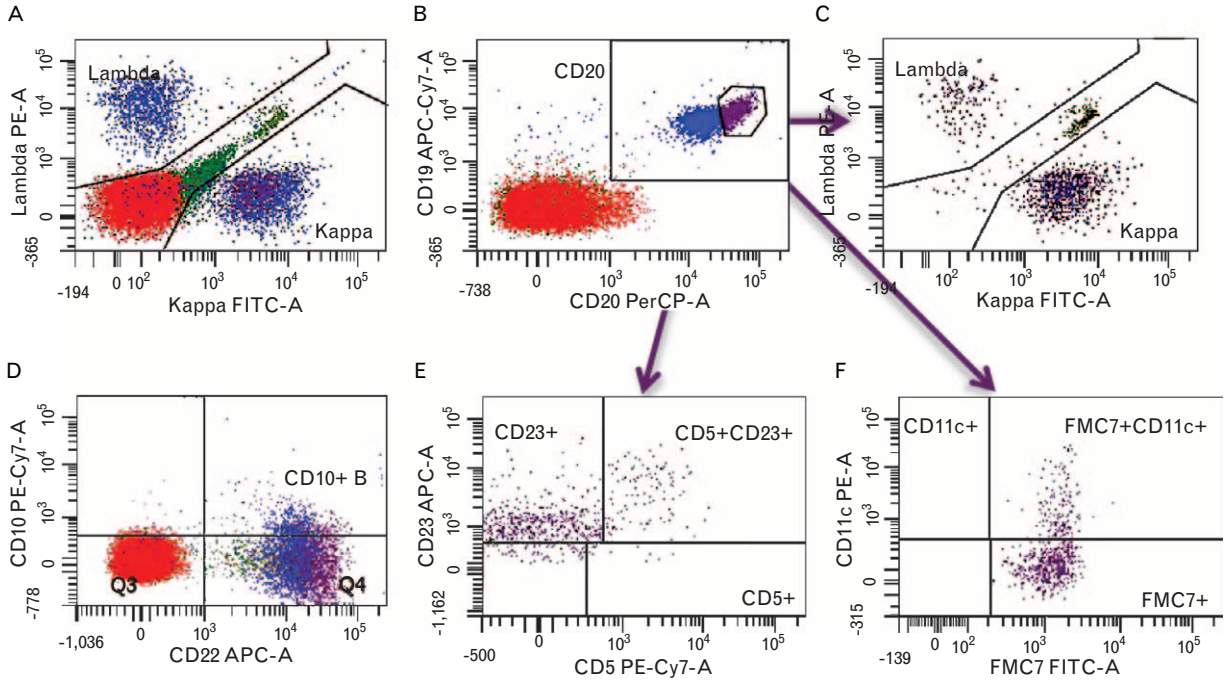

Fig. 4 Eight-colour analysis reveals a small B cell population. Lymphocytes in a peripheral blood sample were gated on CD45 and side scatter. Bright CD20$^+$ cells are displayed purple, moderate CD20$^+$ cells are blue, T cells (CD3$^+$) are red, and non-B non-T cells (mostly NK cells) are green. Panel A shows κ versus λ for all lymphocytes. Panel B shows selection of the bright CD20$^+$ B cells; their κ versus λ characteristics are shown in Panel C. Panel D shows other markers in the same tube as Panels A–C. Panels E and F are from a separate tube and show only the bright CD20$^+$ cells.

clonal T cells can be directly determined, and control samples do not demonstrate Vβ restriction.[17,18] Furthermore, the approach has been validated by Vβ sequence analysis,[15] and is useful in the assessment of minimal residual disease, when only a single Vβ antibody is required.[17]

Flow cytometry enables identification of the abnormal cell population when normal T cells are present. In a study with four-colour flow cytometry, potentially abnormal populations were selected either by the standard markers CD3 and CD4 or CD8, or by markers aberrantly expressed by the abnormal cells. Selection on the basis of aberrant tumour-specific markers was significantly more effective in identifying clonal populations.[17] These authors suggested that the strategy may be even more effective with higher numbers of colours. Such an approach is illustrated in the bone marrow sample displayed in Fig. 5. Initially the lymphocytes were assessed with an eight-colour panel containing CD45 for gating and seven other markers: CD3, CD4, CD5, CD7, CD8, CD16 and HLA-DR. In this case, an unusual $CD8^+$ T cell population was identified on the basis of dim expression of CD5 (Fig. 5A). In the Vβ analysis, CD45, CD3, CD5 and CD8 were used to select this population, and two fluorochromes were allocated to Vβ in each of a series of tubes to assess clonality.[17] The majority of the $CD45^+$, $CD3^+$, dim $CD5^+$ and $CD8^+$ cells were $V\beta22^+$ (Fig. 5B). By contrast, only low numbers of these cells were positive for any of the other Vβ specificities; a representative example is shown in Fig. 5C. The T cells other than the abnormal dim $CD5^+$ population were polyclonal by Vβ analysis (data not shown).

This example demonstrates the capacity of PFC to identify and efficiently characterise an abnormal monoclonal T cell population of substantial size, that could otherwise have been missed because of the presence of normal polyclonal T cells. In many cases, enhanced detection of monoclonal T (or B) cell populations is clinically beneficial. A disadvantage of PFC, however, is its increased capacity to identify small monoclonal populations of uncertain clinical significance.

Plasma cell disorders

The role of flow cytometry in plasma cell disorders is less well established than in leukaemias and lymphomas, and plasma cell disorders were not included in previous reviews of flow cytometry in this journal.[1,2] However, evidence has emerged over the last several years that flow cytometry can make a substantial contribution to the laboratory assessment of plasma cell disorders, and therefore this topic is covered in depth in this review. An important reason for the slow acceptance of flow cytometry in plasma cell disorders is that flow cytometry substantially under-estimates the percentage of neoplastic plasma cells compared with microscopy. In multiple myeloma, flow cytometry may only detect approximately one-quarter as many cells as morphology.[19,20] Such under-estimation of plasma cells has caused skepticism among clinicians. Several reasons have been proposed to explain this discrepancy. Samples used for morphology are often the 'first-pull' in a bone marrow aspirate, whereas samples sent for flow cytometry may be subsequent samples, with a higher amount of blood contamination than the first-pull sample.[20,21] Plasma cells may adhere to lipid-enriched spicules that are represented in bone marrow smears but not in the bone marrow fluid that is prepared into a cell suspension for flow cytometry.[19] Processing and analysis of the sample by flow cytometry may lead to loss of plasma cells.[20] Plasma cell populations that express CD56 are more seriously under-estimated by flow cytometry than those

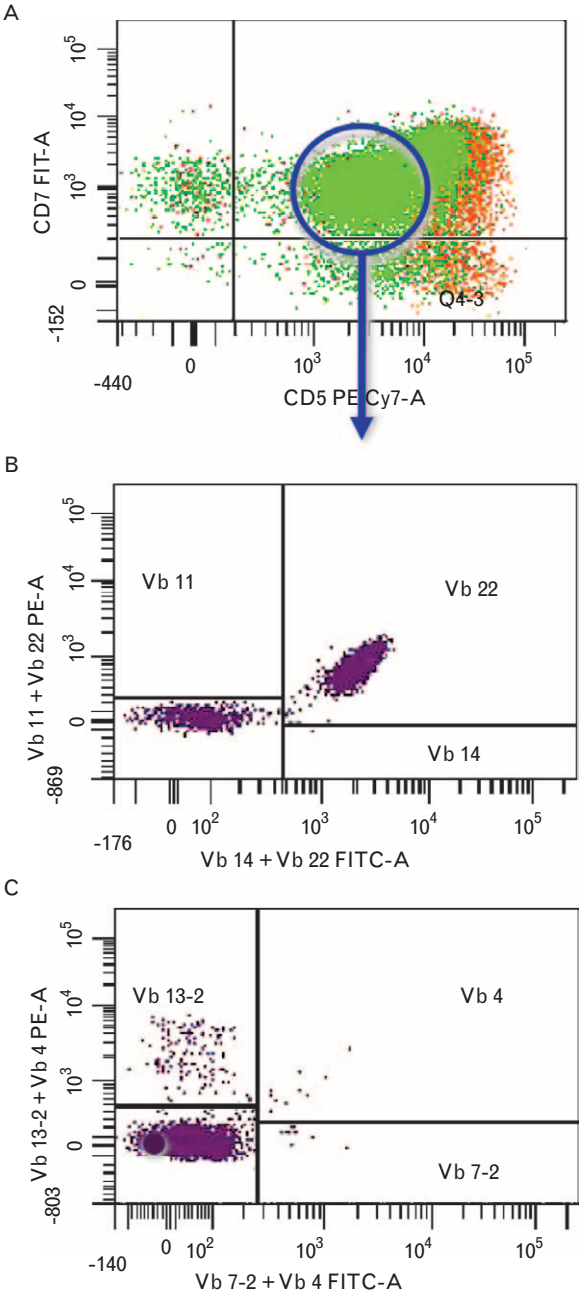

Fig. 5 Assessment of a T cell population by eight-colour cytometry. Lymphocytes in a bone marrow sample were gated on CD45 and side scatter, and stained with a T cell panel. In Panel A, $CD3^+CD4^+$ cells are orange and $CD3^+CD8^+$ cells are green. A suspicious $CD3^+$, $CD4^-$, dim $CD5^+$, $CD8^+$ population was selected (blue circle) and stained with Vβ markers. This population only is shown in Panels B and C. The majority of these cells are $V\beta22^+$, and only small numbers stain for other Vβ markers.

lacking CD56.[22] CD56 is a cell adhesion molecule that is capable of homotypic adhesion. Therefore, CD56-based cell-cell adhesion may cause plasma cells to form doublets, resulting in an under-estimation of CD56 positive cells by flow cytometry.[22]

The figure of 10% of plasma cells in bone marrow microscopy has been used as a cut-off between multiple myeloma and monoclonal gammopathy of uncertain significance (MGUS).[23] It has been proposed that flow cytometry could use a lower figure for such a cut-off. It has been argued that the percentages determined by flow cytometry do correlate with those from

morphology, and are based on larger numbers of cells. Therefore, some have argued for the use of flow cytometry to enumerate plasma cells.[21] However, in another report, the extent to which flow cytometry under-estimates plasma cells has been shown to be highly variable, and no single cut-off value reliably distinguished between MGUS and multiple myeloma.[22] Therefore, it seems unlikely that flow cytometry will replace microscopy in determining the percentage of plasma cells for the diagnosis of multiple myeloma.

Nevertheless, flow cytometry can make a number of other contributions in the assessment of plasma cell disorders. The combination of CD45, CD38 and CD138 provides an excellent strategy to identify plasma cells.[24] CD45 is useful to identify the major leukocyte populations, and is often dimmer on neoplastic plasma cells than on their normal counterparts.[25] CD38 is expressed on a variety of haematopoietic cell types, but its intensity is greater on plasma cells than on other cell types.[3] By contrast CD138 is a more specific marker of plasma cells, as it is not expressed on other haematopoietic cells, although CD138 is not as sensitive in identifying plasma cells as CD38.[3]

Although plasma cells down-regulate surface immunoglobulin, they contain abundant intracellular immunoglobulin. Intracellular staining readily detects their κ or λ light chains, and is a powerful method to assess plasma cell clonality.[24] When B cells differentiate into plasma cells, they normally down-regulate several cell surface molecules including CD20, but retain CD19 expression. By contrast, neoplastic plasma cells are usually negative for CD19. Plasma cells in the majority of multiple myeloma cases are CD56 positive, whereas CD56 is not usually expressed on normal plasma cells.[21,26] Other markers that may contribute to distinction between normal and neoplastic plasma cells are CD28 and CD117, which are absent on normal cells and present on some cases of neoplastic plasma cells.[27] CD27 is present on normal plasma cells but is down-regulated in some cases of neoplastic plasma cells.[28]

PFC enhances the capacity of flow cytometry to evaluate multiple extracellular and intracellular markers in a single plasma cell. An effective way of assessing plasma cells is to combine the following markers in a single tube: CD45, CD38 and CD138 (for identification of plasma cells), κ and λ (for clonality), CD19 and CD56 (for abnormal plasma cells as described above) and CD20 (expressed on about 10% of cases of plasma cell neoplasms).[29] Assessment of CD20 in multiple myeloma may be useful in that there are reports of good responses to therapy with anti-CD20 antibodies in selected individuals with $CD20^+$ multiple myeloma.[30]

An example of the utility of this eight-colour strategy is presented in Fig. 6. The plasma cells are identified as $CD38^+$ and $CD138^+$ (Fig. 6A). Surface CD19 and CD56 staining (Fig. 6B) revealed two major populations, a $CD19^+$, $CD56^-$ population which was polyclonal by intracellular κ and λ staining (Fig. 6C), and a $CD19^-$, $CD56^+$ population which was predominantly λ (Fig. 6D). This example demonstrates that PFC is an effective means of comprehensively analysing plasma cells, and identifying a clonal plasma cell population in the presence of polyclonal plasma cells. A comparison between a four- and a six-colour panel for the evaluation of plasma cells has been published. This report used a single six-colour tube, containing antibodies to CD38, CD138, CD19, CD56 and cytoplasmic κ and λ. (All six markers in this tube are included in the eight-colour tube in Fig. 6.) The six-colour tube was significantly more effective than four-colour cytometry in detecting small clonal populations of plasma cells.[31]

Flow cytometry is effective in assessing non-secretory multiple myeloma, and in distinguishing lymphoplasmacytic lymphoma and other B cell lymphomas from multiple myeloma.[24] If a monoclonal population is detected, the combination

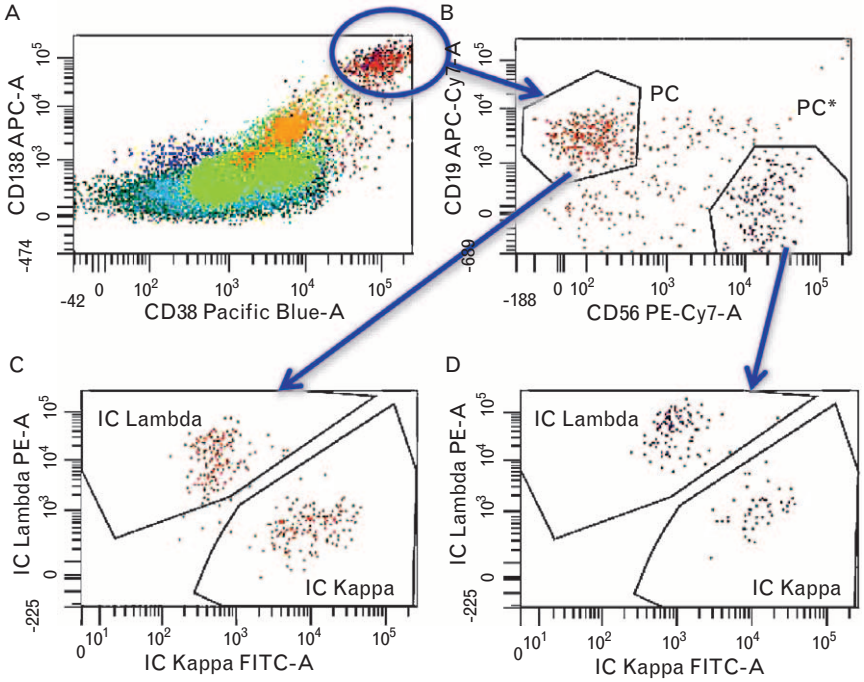

Fig. 6 Eight-colour flow cytometry of plasma cells. Panel A, plasma cells in a bone marrow sample were identified with CD38 and CD138. Panel B, the plasma cells are assessed for CD19 and CD56 and largely fall into two populations. Their intracellular κ versus λ plots are displayed in Panels C and D. The $CD19^+CD56^-$ plasma cells (Panel C) are essentially polyclonal, whereas the $CD19^-CD56^+$ plasma cells (Panel D) are largely λ^+.

of $CD19^+$ and $CD20^+$ is more likely to be present on a B cell lymphoma than a plasma cell disorder, and such cells should be checked for CD138, which is not expressed in lymphomas.[3] Flow cytometry has been used to assess the likelihood of progression of MGUS to multiple myeloma. In MGUS, if >95% of plasma cells are a clonal population, there is increased risk of progression.[32,33] In multiple myeloma, the combination of CD28 positivity and CD117 negativity has been associated with shorter progression-free survival.[34] The role of flow cytometry in assessing minimal residual disease in plasma cell disorders will be reviewed in the following section.

Monitoring of minimal residual disease

The term minimal residual disease (MRD) is used in the context of the treatment of haematological malignancy to describe the presence of residual disease that is not detectable by conventional microscopy, but can be determined by immunophenotypic, molecular or cytogenetic means. Numerous studies have now demonstrated that MRD is a powerful prognostic indicator in a number of conditions. The most conclusive evidence is available for childhood acute lymphoblastic leukaemia (ALL), where many reports demonstrate that MRD detected during the first 2–3 months of treatment is the strongest predictor of relapse.[35] Over 90% of ALL cases have leukaemia-associated phenotypes that can be defined by flow cytometry at diagnosis, then monitored during treatment.[36] Different protocols require a minimum of between 20 and 100 cells to define an MRD population.[37,38] Detection by flow cytometry at the level of 0.01% is standard, requiring analysis of 2×10^5–10^6 leukocytes. Chemotherapy can cause changes in the immunophenotype of ALL cells that may complicate the assessment of MRD, but ALL cells can still generally be distinguished from normal cells.[39]

In ALL, MRD can also be determined by polymerase chain reaction (PCR) assessment of antigen receptor genes or leukaemia-associated translocations. Most cases of ALL (both B and T lineage) have rearranged antigen receptor genes (IG or TCR).[35] At the level of 0.01% MRD detection, flow cytometry and PCR give concordant results in the vast majority of cases.[40] For determination of the number of malignant cells in MRD, flow cytometry is more suitable than PCR. In large studies of childhood B-lineage ALL, a higher percentage of cases have been successfully assessed by flow cytometry than by PCR. In a study of 2143 children, four-colour flow cytometry was performed in a single institution. MRD was successfully evaluated at a detection level of 0.01% in 92% of patients. In a further 4%, the sample cellularity was low or the immunophenotype of the malignant cells was not sufficiently different from normal cells, and MRD could only be assessed at the less sensitive level of 0.1%. The MRD result was indeterminate in only 1.4% of cases, and no sample was provided in the remaining 2.7%.[37] In another large study on childhood ALL, adequate data for MRD by PCR were obtained in 78% of patients.[41] The use of both flow cytometry and PCR enables assessment of almost all patients.[35]

In acute myeloid leukaemia (AML), it has been challenging to use pre-treatment markers to predict which patients are likely to relapse.[42] Therefore, there has been increasing interest in assessment of MRD, because numerous studies indicate that MRD is a good predictor of prognosis.[43] Furthermore, a recent study has used MRD, determined by flow cytometry, as the basis for risk directed therapy. The study, on childhood AML, found that the outcomes of the MRD-informed approach were superior to those of historical controls.[44]

However, MRD assessment in AML by flow cytometry has not yet widely entered into routine practice.[42] Compared with ALL, MRD determination of AML by flow cytometry is more complex. The intensity of expression of standard markers in an AML population is often more variable.[45] Furthermore, there is a wider range of leukaemia-associated phenotypes (LAPs) in AML. It is recommended to search for a LAP at the time of diagnosis with a wide panel of antibodies. If a LAP is found, then it can be assessed in follow-up samples after the initiation of therapy.[45] In AML, immunophenotypic differences have been reported between samples at diagnosis and relapse in most cases, and a broad panel of antibodies has been recommended to assess MRD.[46] However, a more recent report indicated that such differences did not interfere with MRD analysis.[47]

Flow cytometry may detect AML cells at a level of 0.1–0.01% of bone marrow leukocytes.[42,48] Earlier studies indicated that 60–88% of AML patients have a LAP that can be used to monitor MRD.[45] One study compared four-colour with six-colour flow cytometry, and found that the six-colour system considerably improved the capacity of flow cytometry to identify LAPs on AML cells.[49] A study on a small number of AML patients showed that eight-colour flow cytometry identified a LAP in all six patients, whereas three-colour cytometry only found a LAP in three of the six patients.[50] These studies suggest that flow cytometry with high numbers of colours may make a useful contribution to MRD assessment in AML.

In chronic lymphocytic leukaemia (CLL) patients who need therapy, it was established a decade ago that MRD is a predictor of outcome. MRD can be assessed by flow cytometry at a sensitivity of 0.01% of leukocytes.[51] Subsequent research has resulted in an international standardised four-colour flow cytometry approach, with a high level of accuracy and low inter-laboratory variation. Flow cytometry is considered more suitable than molecular testing for routine analysis, because of the wider availability of the technology, faster turn-around time and lower cost, coupled with satisfactory sensitivity. Furthermore, analysis of peripheral blood is suitable in most cases.[52] However, molecular analysis by PCR, based on allele-specific oligonucleotides (designed on patient immunoglobulin heavy chain gene sequences), is more sensitive than flow cytometry.[52,53]

In multiple myeloma, the presence of circulating myeloma cells in the peripheral blood prior to stem cell harvest was associated with lower survival and decreased time to disease progression.[54] A recent study investigated treated multiple myeloma patients who were defined as being in complete remission on the basis of <5% plasma cells in the bone marrow by microscopy, absent paraprotein on immunofixation, and normalisation of serum free light chain ratio. The progression-free survival of these patients was significantly longer if their multiple myeloma cells were undetectable by flow cytometry.[55] Flow cytometry techniques have been established to detect abnormal plasma cells at a frequency of 0.01%. Detection of minimal residual disease by flow cytometry 3 months after transplantation was strongly correlated with early disease progression.[56] The number of circulating abnormal plasma cells at the time of diagnosis of multiple myeloma, determined by flow cytometry, has been correlated with a poorer outcome.[57]

Paucicellular samples

Apart from MRD, another example of rare event detection by clinical flow cytometry is analysis of samples that typically contain small numbers of cells, such as cerebrospinal fluid (CSF). PFC makes it possible to obtain more information when there is only sufficient sample for one or a small number of tubes. With CSF samples, unless an initial leukocyte count reveals a high cell number, it is prudent to commit all the cells in the sample to a single tube.[58] Therefore, the higher the number of markers, the more information will be gained from the one tube. Studies have shown flow cytometry improves the detection rate of CSF involvement of haematopoietic neoplasms.[59] If the patient has not previously been diagnosed, the CSF sample should be tested with markers covering a broad range of lineages. However, if the patient has already been diagnosed, it is important to tailor the markers to the known malignancy.

Circulating tumour cells

There is accumulating evidence that assessment of circulating tumour cells (CTC) in patients with carcinoma may be useful to predict survival and response to therapy.[60,61] In metastatic cancer, CTC are present at the order of one CTC per 10^5–10^7 leukocytes,[60,62] so it is challenging to find enough cells to demonstrate CTC persuasively and to provide reliable quantitation. Flow cytometry may have a role in assessment of CTC, although it must be recognised that CTC are being identified by many different strategies, both cellular and PCR-based.[63] Cellular assays can identify CTC by general epithelial lineage markers such as EpCAM and cytokeratins, or by markers with more restricted expression such as HER-2/neu.[64] While clinical flow cytometry has been optimised for leukocyte immunophenotyping, flow cytometry can also be used to assess cells of epithelial origin, for example carcinoma cells in effusions.[65] The Human Leukocyte Differentiation Antigen (HDLA) workshops have been included in the Human Cell Differentiation Molecules (HCDM) organisation to allow allocation of CD numbers to non-haematopoietic markers including epithelial markers.[66]

Amongst the various assays that have been developed to assess CTC, an advantage of flow cytometry is high specificity. It can assess CTC on the basis of multiple extracellular and intracellular markers.[67] In addition, DNA content, cell size and cell viability can be determined. With any immunophenotyping strategy, there is a limitation that carcinoma cells may down-regulate expression of epithelial markers, and thus may not be detected.[68] Furthermore, tumour-specific markers exhibit heterogeneity between individuals, and also between cells within the one individual.[64] However, the risk of false negative results in the detection of CTC can be reduced by including a range of epithelial and tumour markers.[60] PFC has the potential to assess a greater range of such markers. Another problem with cell-based assays is that leukocytes, especially when activated, may express epithelial cell markers.[69] Assays for CTC may exclude leukocytes on the basis of staining with the pan-leukocyte marker CD45, although care needs to be taken to ensure epithelial cells are not excluded because of non-specific staining for CD45.[70]

Flow cytometry is limited by the number of events that can be examined. Current clinical cytometers can take ~60 seconds to count 10^5 cells; these instruments would take 16.7 hours to count 10^8 cells. Because of the rarity of CTC, an enrichment step is generally used before flow cytometry to increase the likelihood of finding the desired cells. Several technologies are available to concentrate cells, including immunomagnetic beads, density gradient separation and cell size-restrictive filtration. In a recent report, CTC were concentrated with EpCAM-labelled immunomagnetic beads, followed by enumeration by flow cytometry using EpCAM to identify epithelial cells and CD45 to exclude leukocytes.[70] In a more elaborate strategy, patients were subjected to leukapheresis to obtain $>10^{10}$ mononuclear cells. Next, CTC were enriched by elutriation, and finally they were identified by flow cytometry on the basis of $EpCAM^+$ and $CD45^-$.[71] An alternative approach, that has been demonstrated in mice, is intravenous injection of a tumour-specific fluorescent ligand followed by intravital flow cytometry.[72] Advances in information technology now enable analysis of very large data files, so millions of events can be collected and properly analysed to identify CTC.

IMPLEMENTATION AND MAINTENANCE OF QUALITY

Choosing a system

Flow cytometry has benefited from advances in technologies in many areas. Lasers are improving, optics and fluidics systems are more stable, computers are more powerful and instruments are getting smaller.[73,74] Many new fluorochromes are commercially available, and techniques for conjugating them to antibodies have improved. Robotic sample preparation systems, that are more efficient and accurate, are being introduced into clinical cytometry. All these factors make the decision of the most appropriate system more complex.

Although the use of increased colours improves many aspects of laboratory operations,[75] it also increases the resources and expertise needed for development, maintenance and quality control. Once a laboratory introduces a more complex system, staff require more training and need to achieve a higher understanding of gating and troubleshooting. When choosing a new system, laboratories should match the system with the expertise and motivation of the staff.

The first step in choosing a flow cytometry system is to assess the usefulness of each level of increase in colours. Another consideration is that CD45 gating is highly recommended for oncology immunophenotyping,[4,76] so CD45 should be included in all panels. This means that a four-colour system effectively provides three colours for lineage and descriptive markers. Table 1 shows a process for deciding the benefits of extra colours, starting with a five-tube, four-colour lymphocyte panel. With a five-colour system, only four tubes would be needed to run the same markers. With six colours, four tubes are still required, but extra spaces allow room for both CD19 and CD20 as backbone markers in every B cell tube, as well as an extra marker in the T cell tube. With seven colours, the markers now fit into three tubes and there is an additional space in the T cell tube. With nine colours, the original markers fit into two tubes. With 12 colours, all the B cell markers fit into one tube. The laboratory should determine how the various numbers of colours would affect its operations, and compare these impacts to the available instrumentation.

Laboratories must decide whether to make a single large jump to an instrument with the current maximum number of colours, or take several smaller steps. If moving in small steps, it may still be useful to acquire an instrument with full capabilities, so that each step can be performed on the same

Table 1 Increasing the number of colours reduces the number of tubes

No. colours	Tube number	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10	FL11	FL12
4	1	CD45	CD20	sIg κ	sIg λ								
	2	CD45	CD3	CD4	CD8+CD16/56								
	3	CD45	CD19	CD5	CD38								
	4	CD45	CD22	CD23	CD10								
	5	CD45	CD19	FMC7	CD11c								
5	1	CD45	CD20	sIg κ	sIg λ	CD10							
	2	CD45	CD3	CD4	CD8	*CD16/56*							
	3	CD45	CD19	CD5	CD23	CD38							
	4	CD45	CD19	CD22	FMC7	CD11c							
6	1	CD45	CD20	sIg κ	sIg λ	CD10	*CD19*						
	2	CD45	CD3	CD4	CD8	*CD16/56*	*CD7*						
	3	CD45	CD19	CD5	CD23	CD38	*CD20*						
	4	CD45	CD19	FMC7	CD11c	CD22	*CD20*						
7	1	CD45	CD20	sIg κ	sIg λ	CD10	FMC7	CD11c					
	2	CD45	CD3	CD4	CD8	*CD16/56*	*CD7*	*CD19*					
	3	CD45	CD19	CD5	CD23	CD22	CD38	*CD20*					
9	1	CD45	CD20	sIg κ	sIg λ	CD10	CD5	CD23	CD22	CD38			
	2	CD45	CD3	CD4	CD8	*CD16/56*	*CD20*	CD19	FMC7	CD11c			
12	1	CD45	CD20	sIg κ	sIg λ	CD10	CD19	CD5	CD38	CD22	CD23	CD11c	FMC7
	2	CD45	CD3	CD4	CD8	*CD16*	*CD56*	*CD7*	*TCRγδ*	*CD19*	*CD5*		

A four-colour five tube screening panel contains 16 different markers. When the number of colours increases, these markers can be analysed in fewer tubes. Other improvements, such as the separation of CD8 and CD16/CD56, and the inclusion of CD19 and CD20 in B cell tubes, are indicated in italics.

instrument. The next and much larger decision is choice of instrument. This decision needs to consider specific instrument model reputation, manufacturer reputation, on-going technical support, on-going engineering support, antibody range and quality, and software (acquisition and analysis).

Development of antibody panels

When investigating cells, panels need to include lineage marker(s) to assign cells to the correct lineages, and descriptive markers to obtain more precise information.[77,78] There are two strategies, either to use small screening panels that trigger more extensive lineage-specific panels, or to use larger panels initially, to enable full assessment in the first instance.[79] The former approach can significantly reduce antibody costs, but the latter approach simplifies laboratory operations, significantly decreasing turn-around time, and reducing staff time handling each sample. If small screening panels are used, experienced staff need to be available to assess for abnormalities as soon as the data from the screening panels are analysed, so that further markers can be tested before the sample deteriorates.

There are many factors to consider for panel design, including frequency of use, clinical implications of results, required sensitivity, antibody-fluorochrome stability and lot to lot consistency, and stability in pre-mixed 'cocktails'. For a given channel, it is recommended to use the same fluorochrome in all tubes rather than using similar but not identical fluorochromes from tube to tube. For example, FITC and AF488 have similar properties, but one or the other should be used in all tubes. Because each combination of fluorochromes requires specific settings, using the same fluorochromes throughout will mean fewer settings to create and maintain. As systems become more complex, it becomes more important to simplify maintenance by such strategies.

Basic panel construction theory recommends that the brighter fluorochromes be used for the lower density antigens.[79] However, the matching of antibodies to fluorochromes in a clinical laboratory depends most on practicalities and cost effectiveness. Panels will be constructed to target specific neoplasms, and then commercially available antibody-fluorochrome combinations will be selected to fit. Using the current antibodies in new panels will make correlation with the current system easier. The newer dyes not used in four- and five-colour systems tend to be more expensive, thus it is not cost effective to test many alternative antibodies. Consequently, laboratories will tend to look to their peers, quality assurance programs and standardisation publications to guide choices. However, it is important to consider that if a laboratory will use a panel for many years, then spending more on evaluation will be worthwhile. Antibody panels that we have validated for eight-colour analysis are listed in Table 2.

The pre-mixing and storage of large batches of antibodies ('cocktailing') is increasingly important with higher numbers of colours, where accidental omission of an antibody may be more difficult to identify.[80] Cocktailing reduces set-up time and ensures that every antibody is added, providing confidence that an unusual negative result is a true finding. Creating in-house cocktails requires more expertise, and may take several attempts to optimise. Cocktails need to be tested for stability, which is especially important with tandem dyes.[75,81] We have

Table 2 Validated eight-colour panels

	PacB	PacO	FITC	PE	PerCP	PE-Cy7	APC	APC-Cy7
L1	CD3	CD45	sIg κ	sIg λ	CD20	CD10	CD22	CD19
L2	CD3	CD45	CD7	CD16	CD8	CD5	CD4	HLA-DR
L3	CD38	CD45	FMC7	CD11c	CD20	CD5	CD23	CD19
M1	CD38	CD45	CD34	CD110	CD33	CD10	CD117	CD19
M2	CD14	CD45	CD64	CD36	CD33	CD13	CD71	HLA-DR
M3	CD11b	CD45	CD15	MPO	CD33	CD56	CD123	HLA-DR
PC	CD38	CD45	icIg κ	icIg λ	CD20	CD56	CD138	CD19

The table shows validated eight-colour panels for three lymphocyte tubes (L1, L2 and L3), three myeloid tubes (M1, M2 and M3) and one plasma cell tube (PC). APC, allophycocyanin; Cy, cyanine; FITC, fluorescein isothiocyanate; icIg, intracellular immunoglobulin; L, lymphocyte; M, myeloid; PacB, Pacific Blue; PacO, Pacific Orange; PC, plasma cells; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein complex; sIg, surface immunoglobulin.

validated the panels shown in Table 2 for stability in cocktails for at least 6 weeks. Commercial cocktails can be very useful. The supplier is responsible for the mixing, validating, and stability. The main drawbacks are that commercial cocktails are more expensive than individual antibodies, and must be ordered in bulk, and the antibodies and fluorochromes will only be from the one supplier, limiting optimisation and experimentation.

Compensation

In flow cytometry, the term compensation applies to the process of correction for fluorescence spillover, that is removal of the signal from any fluorochrome from all detectors except the one intended.[82] Compensation becomes more complicated as the number of colours increases.[83,84] Many software packages can auto-calculate compensations,[82] and simplify post-acquisition corrections, but the laboratory staff still need to understand the basic principles of compensation to use these packages effectively, and it is important for operators to be able to manually derive all settings. Compensation can be performed by labelling either cells or antibody capture beads with the antibody of interest.[9,77,82,85] Cells have the advantage that any background factors are taken into account, and forward scatter and side scatter can be set at the same time. However fresh cells are needed, and some markers are not prominently expressed, for example there are too few CD103 positive cells in peripheral blood for compensation. Antibody capture beads are simple to use, but may not capture all antibodies.[77] Also beads do not fully reproduce the cellular environment where the compensation is used. There are many arguments for beads versus cells, but both are effective if handled properly.

Regardless of how the settings are done, it is better to have fewer settings to update. If a clinical instrument has the optics adjusted during scheduled preventative maintenance or laser replacement, the settings must be checked and often re-created. The fewer settings used, the faster they are to re-create, thus reducing instrument downtime. This issue is even more pertinent for laboratories that optimise their compensation settings each day.

Quality control

Quality Control (QC) is a most important aspect of any laboratory. Comprehensive panels and complex analysis mean nothing if the laboratory is not completely confident about the accuracy and reproducibility of its results. All aspects of the system must be monitored in some way, including the sample quality, the antibodies, the assay process, the reagents, the incubation times and conditions, instrument set up, instrument stability, gating algorithms, analysis protocols, report generation and reporting mechanisms.[75,86]

The most common way to perform daily instrument calibration and monitoring is to use standardised latex beads. Newer instrumentation may have extra parameters to monitor, for example extra lasers mean more time delay checks. A cellular control sample, whether in-house or commercial, should also be set up daily, to cover QC for bench processes.[77] It is important to graph the data daily to monitor for instrument drift over time, as well as seeing the range of normal variation. Some manufacturers provide software to automatically graph data and flag when performance is out of range. However, it is important to fully understand what triggers these flags, and to ensure that they will be sensitive enough to detect changes before there are significant effects on clinical results.

The basis of flow cytometry analysis is pattern recognition, so abnormal patterns from assay/reagent errors should be excluded. Almost every antibody used routinely can be checked against expected positive and negative internal controls in normal peripheral blood.[87] This can confirm that markers have stained successfully, thus demonstrating that unusual patterns are true and not artefact. Another valuable intra-assay check is whether the same and similar antibodies give the same results in different tubes. Of course, participation in external quality assurance programs is mandatory for clinical laboratories. Electronic storage of flow cytometry plots in portable document format (PDF) form reduces file size, and makes it simple for a laboratory to quickly review a patient's previous phenotype. Such direct comparison is very useful to assess MRD.

Informatics

Traditionally, analysis software has been provided by the manufacturer of the flow cytometer. However, the complexity of analysis is increasing in PFC, and specialist software companies are playing a greater role. There are now many options available for analysis software, with computers using complicated algorithms to identify and characterise populations. Because the datasets are becoming larger as more cells need to be analysed to see more populations with fewer cells in each, and the software packages become more complex, laboratories need powerful computers.

The increased number of colours in PFC gives information that is hard to express in traditional graphic form.[77] Standard graphs and gates are only two-dimensional; gates can be combined in Boolean formulas, but each region still has to be adjusted in two dimensions. The number of graphs required to display each colour against each other in traditional two dimensional dot plots rises sharply with increasing numbers of colours. For example, to display all the possible comparisons from four colour analysis, six plots are required. With eight colours, 28 such plots are needed, and that is before gated populations are displayed. Thus, it is important to decide the best way to view the information on as few plots as possible. One simple way to track cells on different 2D plots from the one tube is with colour schemes. For example, in Fig. 4, B cells are dark blue, bright $CD20^+$ B cells purple, T cells red, and NK cells green. Defining the major lymphocyte subsets in this way helps to interpret the κ versus λ plot. This approach can be extended by choosing the same colour scheme to display data from different tubes.

In PFC, there are limitations to the traditional approach of identifying populations by gating. Such analysis is time consuming and interpretation may be subjective. Because of the vast complexity of data that can be generated, much information from multiple dimensions may be lost, as the human brain is most effective integrating information from two dimensions. Even three dimensional plots are not easy to use to find populations, especially if they do not have many cells. Nevertheless, with the appropriate tools, adding more colours can clarify populations. For the next stage of software development, complex algorithms are being developed to define clusters of cells in multi-dimensional space in a way that the traditional approach of sequential gating cannot.

One approach is to use principal component analysis to reduce the dimensions of PFC data to two-dimensional displays. This approach can group patients on the basis of the whole flow cytometric profile, and identify differentially

represented phenotypes in groups of patients.[11] Although this approach is not quantitative, it is suitable for identifying abnormal populations present in haematological malignancies.[88] A principal component analysis approach has been used to classify B lineage malignancies. Reference groups of data files for chronic lymphocytic leukaemia, mantle cell lymphoma and follicular lymphoma were built, and then cases were evaluated against these files; 89% of the cases were correctly assigned.[89] A principal component analysis approach was also used to assess six-colour data from patients with common variable immunodeficiency. The analysis enabled the identification of a subpopulation of transitional B cells that was present in the patient group but absent from the controls.[90] These reports indicate that improved methods of data analysis are essential to enable the full benefits of PFC to be realised.

Standardisation

There are variables at every stage in the process of flow cytometry, including type of instrument, choice of monoclonal antibody, choice of fluorochrome, instrument setup, sample preparation, and data analysis. Nevertheless, flow cytometry achieves good performance across laboratories for assays such as enumeration of peripheral blood lymphocyte subsets. In a review of 10 years of performance of an external quality assurance program, assay variability tended to decline over time, suggesting a beneficial impact of the program.[91] These authors proposed that more extensive standardisation would lead to less variability between laboratories. Flow cytometry results can be less satisfactory in more complicated assays such as diagnosis of haematological malignancy and determination of MRD. In some large studies, matters have been simplified by transporting samples to a single laboratory where all the flow cytometry is performed. However this is not practicable for day-to-day clinical cytometry.

The 2006 Bethesda International Consensus conference recommendations set a number of standards for clinical cytometry laboratories. The recommendations define the clinical circumstances for which flow cytometry assessment is appropriate.[92] The recommendations indicate the cell lineage(s) and the markers that should be assessed for each clinical indication.[78] This paper also contains useful information on the principles of construction of antibody panels, and defines information that is either required or optional in clinical flow cytometry reports. The Consensus also proposes guidelines for the training and education of personnel in flow cytometry.[93] This paper recommends creating a certification program for flow cytometrists, and such a program is now being developed by the International Clinical Cytometry Society and the International Society for Advancement of Cytometry.

Various other international groups have recommended more extensive approaches to standardisation, such as a set of standardised protocols for cell handling in the assessment of myelodysplastic syndromes.[94] To date, the most extensive proposals for standardisation have been put forward by the EuroFlow Consortium. It is developing full standardisation of instrument setup, sample preparation, immunostaining procedures, fluorochromes, and eight-color antibody panels, as well as a novel software system for automated data analysis.[38,89] EuroFlow has performed extensive validation on panels of specific antibody-fluorochrome conjugates for eight-colour analysis (www.euroflow.org). It remains to be seen whether such standardised approaches will be adopted throughout the flow cytometry community.

CONCLUSION

Technological developments in flow cytometry will continue to provide opportunities for increasingly sophisticated analysis of clinical specimens. Evidence is emerging that implementation of PFC offers clinical benefits. There is potential for greater sensitivity in the diagnosis and monitoring of haematological and other malignancies. PFC is very helpful in the assessment of small subsets in the presence of larger populations, and in the assessment of minimal residual disease and paucicellular specimens. An increase in the number of colours is also associated with higher demands in maintaining a quality system. It is essential to give close attention to the many aspects of flow cytometry that need to be mastered in order to produce high quality clinical reports and fulfill the potential of advanced instrumentation.

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