



**HAL**  
open science

# A Review of Flow Cytometry and its Use in the Diagnosis and Management of Mature Lymphoid Malignancies

Ruth de Tute

► **To cite this version:**

Ruth de Tute. A Review of Flow Cytometry and its Use in the Diagnosis and Management of Mature Lymphoid Malignancies. *Histopathology*, 2011, 58 (1), pp.90. 10.1111/j.1365-2559.2010.03703.x . hal-00613813

**HAL Id: hal-00613813**

**<https://hal.science/hal-00613813>**

Submitted on 6 Aug 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**A Review of Flow Cytometry and its Use in the Diagnosis and Management of Mature Lymphoid Malignancies**

Journal:	<i>Histopathology</i>
Manuscript ID:	HISTOP-09-10-0496.R1
Wiley - Manuscript type:	Review
Date Submitted by the Author:	14-Oct-2010
Complete List of Authors:	de Tute, Ruth; St James's Institute of Oncology, HMDS
Keywords:	Flow Cytometry, Lymphoma, Lymphoid Neoplasm, Diagnosis

SCHOLARONE™  
Manuscripts

view

# **A REVIEW OF FLOW CYTOMETRY AND ITS USE IN THE DIAGNOSIS AND MANAGEMENT OF MATURE LYMPHOID MALIGNANCIES**

Ruth M. de Tute

Haematological Malignancy Diagnostic Service, St James' University Hospital, Leeds

## **Abstract**

The last decade has seen major advances in flow cytometric immunophenotyping and this has expanded the utility of flow cytometry to investigate the antigens present on normal and neoplastic haematopoietic cells. This review summarises how flow cytometry is currently used in the diagnosis and management of mature lymphoid malignancies. The establishment of disease-specific phenotypes allows the creation of assays which can detect neoplastic cells with high specificity and sensitivity. Certain lymphoid neoplasms are immunophenotypically well defined whilst others are more heterogeneous. The availability of more sophisticated cytometers and a wider selection of antibodies in routine diagnostic laboratories will lead to the resolution of these more complex disease entities.

## **Use of flow cytometry in the diagnosis of mature lymphoid neoplasms.**

Historically, classification of lymphoma has been heavily reliant upon cytomorphology <sup>(1)</sup>. Recent advances in technology have provided many new investigative approaches and these methods have generated a vast amount of new information about the haematopoietic system and the development and function of cells within it. This knowledge has led to original ways to define disease entities and classification has evolved into a multiparametric process which combines morphological, immunophenotypic and genetic data, as well as considering information regarding clinical presentation and disease course. The W.H.O. Classification of Tumours of Haematopoietic and Lymphoid Tissues, first published in 2001<sup>(2)</sup> and updated in 2008 <sup>(3)</sup>, utilises all this data to categorise haematological malignancies. Whilst some of the more complex and heterogeneous disease entities

still depend upon morphological assessment for diagnosis, other classifications have been defined by the presence of a specific genetic abnormality or a specific immunophenotype. Flow cytometric immunophenotyping is central to the diagnostic strategy laid out for many of the mature lymphoid neoplasms. The International Consensus Recommendations on the flow cytometric immunophenotypic analysis of haematolymphoid neoplasia (Bethesda, 2006) suggests that flow cytometric immunophenotyping is an important part of the investigation in a range of clinical situations, including organomegaly, tissue masses, lymphocytosis and cytopenias<sup>(4)</sup>. As well as being effective in determining presence of disease at presentation and assist with classification, the group concluded that the technique is also effective for staging of a newly diagnosed malignancy, minimal residual disease monitoring and investigation of relapse or progression.

### **The principles of flow cytometry**

Flow cytometry is a technique which allows analysis of the physical properties and patterns of antigen expression in suspensions of intact cells. Preparation of the suspended cells usually involves lysis of any erythrocytes present and staining of the remaining leucocytes with antibodies conjugated with fluorescent dyes (fluorochromes). Once the sample has been incubated, washing is necessary to remove any excess antibody and the cells are then re-suspended prior to acquisition on a flow cytometer. As the fluid is introduced to the flow cytometer the fluidics system of the analyser employs a technique called hydrodynamic focusing to organise cells into a single cell stream. This stream then passes through a number of laser beams of differing wavelengths (this can range from a single laser to four or more depending on the cytometer). As each cell passes a laser beam it disrupts the path of the laser light, scattering it in many directions. This scatter is measured by a number of detectors to generate the parameters of forward scatter (FSC) and side scatter (SSC). These parameters provide information about the size and complexity of cells with, in general terms, FSC being proportional to cell size and SSC relating to the internal complexity or granularity of a cell. The lasers also cause excitation of any fluorochromes which are attached to antibodies bound to the cells and the resulting fluorescence emission is measured using a series of fluorescence detectors and optical filters. Emission spectra of some fluorochromes overlap considerably so mathematical adjustment of the data, termed compensation, is applied to allow for this. Selection of

antibody combinations must be made carefully to minimise the need for compensation correction. The emission data collected indicates presence or absence of antibody-bound fluorochromes. This generates information about the antigens present on the cell to produce a composite immunophenotype.

### **Flow cytometric analysis**

Each measurement taken by a detector is termed an event and each event equates to a single cell. A detector gives every event above the threshold of detection a numerical value so that by the end of acquisition each cell has a set of numerical values representing all the different parameters that have been measured. FSC and SSC are linear variables whilst fluorescence intensities are usually shown on a logarithmic scale. Evaluation of the fluorescent intensities can determine positivity or negativity of protein expression and, due to the quantitative nature of flow cytometry data, it is also possible to gauge strength of expression, usually expressed as mean fluorescent intensity. Results can be represented graphically in two main ways; a single parameter histogram, which is a frequency distribution of the parameter in question, or a two parameter histogram (more commonly known as a dot plot) with one parameter on each axis, each event been shown as a single dot. Examples of both can be seen in Figure 1.

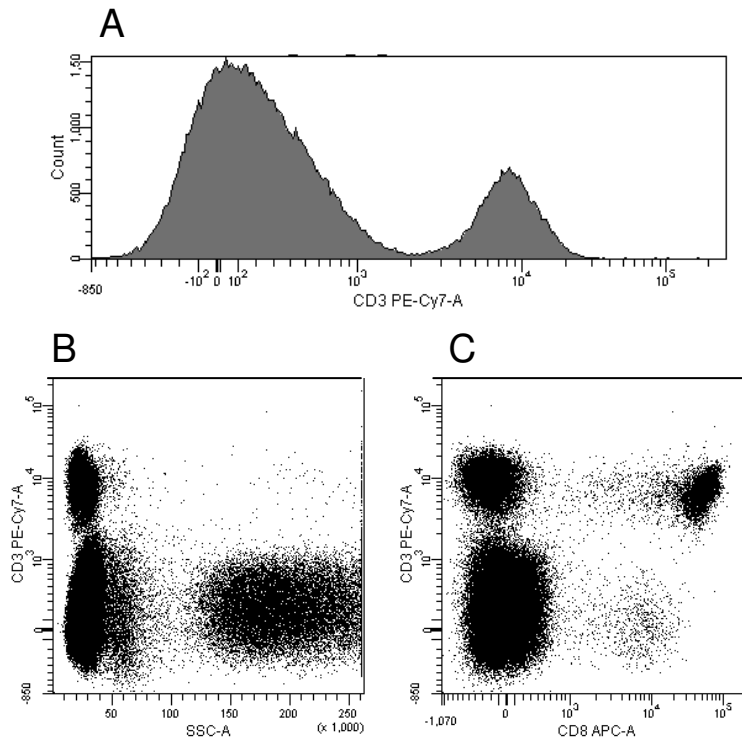


Figure 1. Graphical representation of flow cytometric data.

A is a single parameter histogram for CD3 expression in a normal peripheral blood sample. B and C show the same data in double parameter plots; B demonstrates CD3 expression as compared to side scatter and C shows CD3 vs CD8 expression.

Flow cytometry data is usually analysed by the application of regions and gates, allowing identification of a population of interest and exclusion of other events in further analysis. Gates can be a single region or a combination of multiple regions. Combining of regions is accomplished using the Boolean logic operators AND, OR and NOT (Figure 2 and Figure 3). Once a gate is applied it is possible to examine those cells or events alone to establish a detailed immunophenotypic picture of the population.

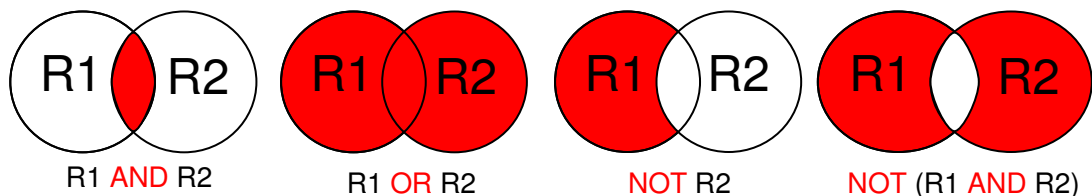


Figure 2. Regions can be combined using the Boolean logic operators AND, OR and NOT to create gates which can be applied during analysis to examine populations of interest in detail. The events which fall in the areas highlighted in red are those which would be included in the gate.

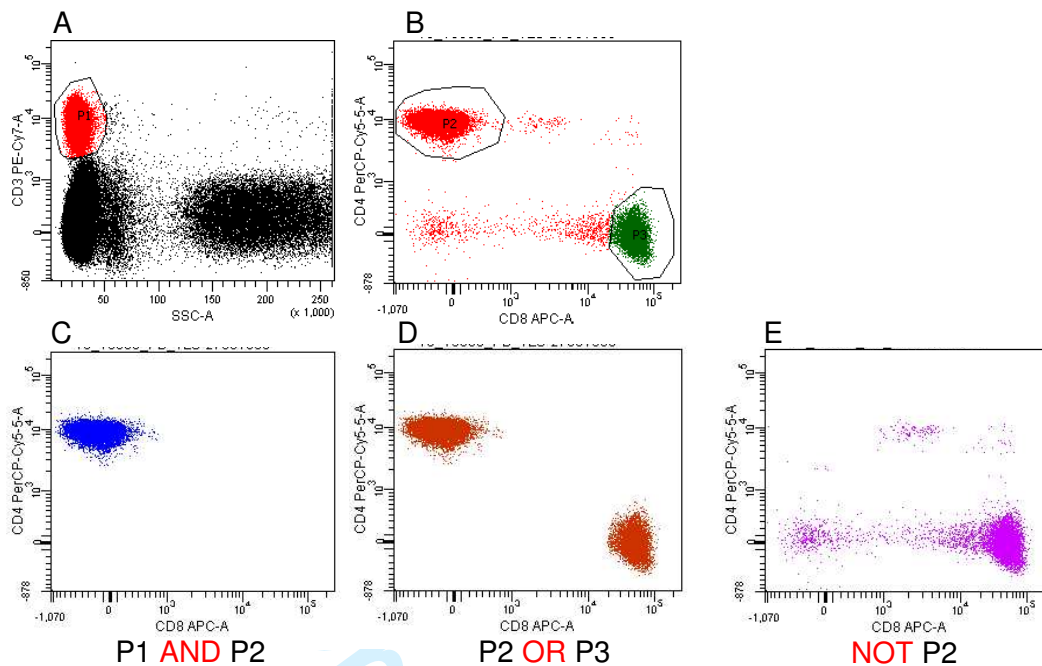


Figure 3. Demonstration of the use of combined regions to interrogate flow cytometric data. A shows a CD3 vs SSC plot for a normal PB sample. A region (P1) has been drawn around the CD3+ events. In B only cells which fall within P1 are shown and a number of subpopulations can be seen. Two further regions have been drawn which apply only to cells in P1: P2 has been created around the CD4+ CD8- events and P3 has been drawn around the CD8+CD4- events. P2 and P3 can then be combined in numerous ways to examine the different sub-populations present. C, D and E show how applying a gate using each of the AND, OR and NOT operators can alter the events which are shown.

### Application of flow cytometry

Early flow cytometers were used primarily for the enumeration of cells and, indeed, the same technology is still used in modern haematology full blood count analysers. Classification of cells became possible following the development of the monoclonal antibody (<sup>5</sup>). By the early 1980's a large number of monoclonal antibodies to human leucocyte antigens had been produced and this revolutionised the field of flow cytometry, allowing flow cytometric immunophenotyping to be used routinely. The last decade has seen major advances in flow cytometric instrumentation and also a substantial increase in the availability of antibodies and fluorochromes. Once confined to research institutions, cytometers with 8 (or more) colours are now available as clinical instruments for use in diagnostic laboratories. It is now possible to routinely assess 10 (or more) parameters simultaneously (<sup>6</sup>). The greater the

number of parameters examined the larger the amount of information which can be obtained from a single preparation of cells. This increase in information has expanded the utility of flow cytometry to investigate the antigens present on normal and neoplastic haematopoietic cells. The immunophenotypic knowledge generated can be applied to determine the presence or absence of normal and neoplastic cell populations of differing types by assessing:

- i. cell lineage
- ii. developmental stage
- iii. presence of clonal populations
- iv. a detailed immunophenotype of the population of interest

Simultaneous assessment of larger numbers of antigens increases the specificity of immunophenotypic classification. This, in turn, increases sensitivity making detection and identification of very small populations within complex mixtures of cells possible.

### ***Flow cytometric approach to the classification of B-cell neoplasms***

The analysis of suspected B-cell neoplasms by flow cytometry proceeds through four well defined steps. The B-cell population is identified and gating established; the stage of B-cell development represented in the sample is assessed; the clonality of the B-cell population is identified and finally a detailed composite phenotype is produced which includes the identification of tumour specific markers.

#### **i) Identification of cell lineage**

The first B-cell antigen to be described was B1 (now known as CD20) and it was first used to characterise malignant cells from lymphoma and leukaemia patients in 1981<sup>(7)</sup>. Since then many more antigens have been identified which are expressed exclusively on B-cells, including CD19, CD22, CD79a, CD79b and other components of the B-cell receptor complex. The most commonly used marker for flow cytometric B-cell gating is CD19 as this marker is present at all stages in the life-cycle of a B-cell, from early progenitors through to terminally differentiated plasma cells. An example of gating with CD19 can be seen in Figure 4.



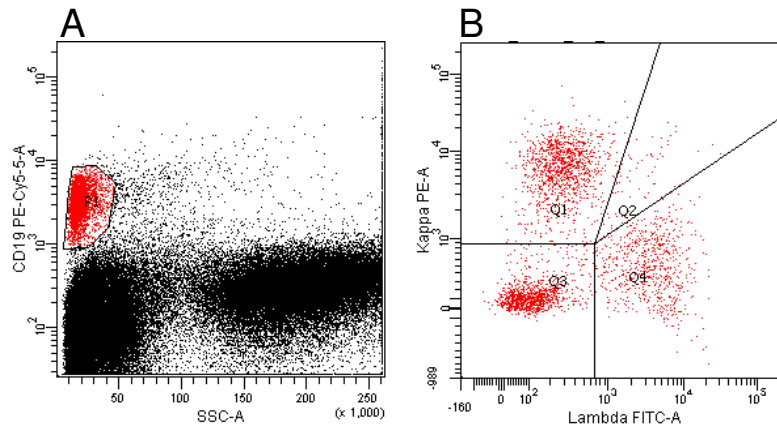


Figure 4. Plot A shows B-cells identified by gating the CD19+ events. CD19 is expressed at all stages of B-cell development from progenitor to plasma cell. Plot B shows that the gated cells do indeed consist of a mixture of Kappa positive and Lambda positive mature B-cells and surface immunoglobulin negative B-cell progenitors.

Some B-cell lymphomas show down-regulation of CD19 and this can make detection of these cell populations problematic using CD19 alone, especially in the context of a background of normal B-cells. Low levels of CD19 expression are most commonly seen in follicular lymphoma but can be seen occasionally in most types of B-cell neoplasm <sup>(8)</sup>. To overcome the gating issues caused by decreased expression it is advisable to use combinations of gating markers, for example CD19 and CD20, as shown in Figure 5.

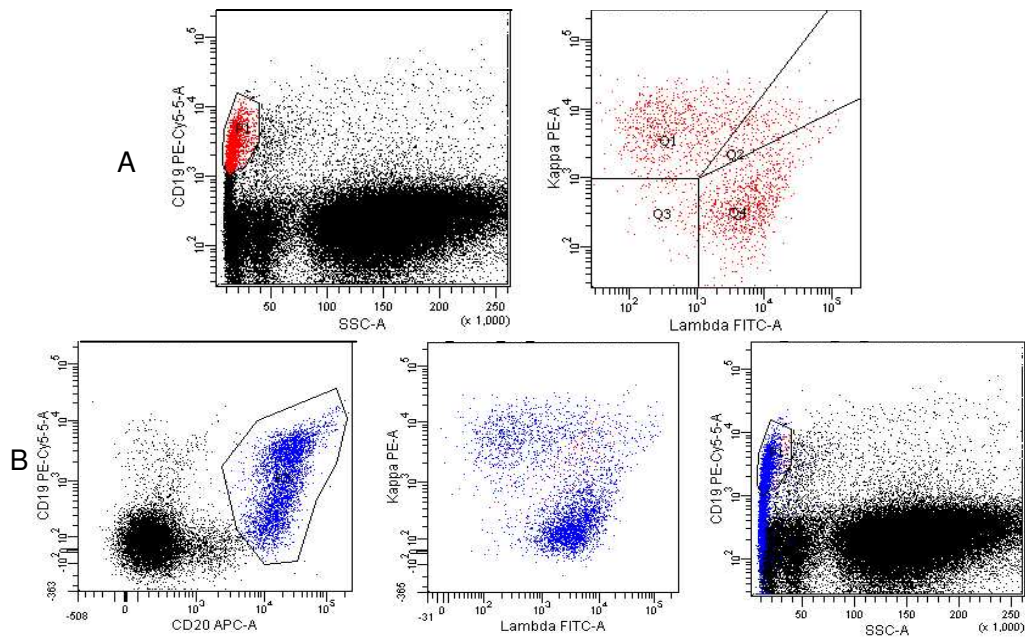


Figure 5. Demonstration of the gating problems encountered when neoplastic B-cells have weak CD19 expression.

Row A shows B-cell gating using CD19 alone. The B-cells comprise 1.9% of total leucocytes and have a slightly skewed kappa:lambda ratio. In Row B, the same sample is gated using CD19 and CD20 in combination. The B-cell proportion increases to 3.8% and there is a clearer lambda positive monoclonal component visible. The final CD19 vs SSC plot shows the B-cells in blue and illustrates the number of cells excluded from the initial gate when CD19 was used alone.

## ii) Developmental stage

Once the B-cell population has been gated as described above, it is possible to obtain information about the stage of cellular development by assessing antigens which have variable expression throughout the life-cycle of the B-cell. One of the most important markers in this respect is CD10. Progenitor B-cells are CD10 positive and express intracellular CD79a/b. They lack CD20 and have no surface immunoglobulin. As the cells mature, expression of CD10 decreases and CD20 increases and the cells gain surface immunoglobulin. Knowledge about the normal differentiation pathways of cells can be used to identify cells which fall outside of the normal pattern.

As with gating, the assessment of developmental stage can be enhanced by using markers in combination. A good example is assessment of CD10 in combination with CD20. Unlike progenitors, neoplastic germinal centre-derived B-cells have strong CD20 expression and so the two populations can be distinguished if the correct

antigens are examined together. The use of CD20 and CD10 in combination to demonstrate a normal regenerative B-cell population is seen in Figure 6.

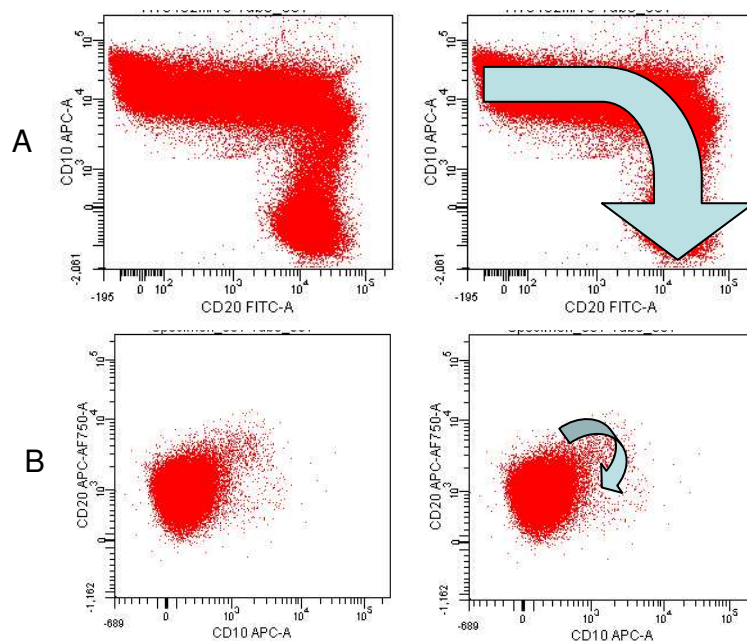


Figure 6. Using CD10 and CD20 to assess developmental stage of B-cells.

The plots in Row A show regenerating B-cells in the bone marrow of a patient following therapy. There are large numbers of progenitor B-cells present which are CD10+CD20-. As the B-cells mature there is a gain of CD20 expression and a loss of CD10 positivity. This pathway of maturation is depicted by the blue arrow in the second plot.

The plots in Row B show normal B-cells in a reactive lymph node biopsy. The majority of the cells have no CD10 expression and are CD20+. A small population of CD10+ events can be seen but these are not progenitors as they have strong CD20 expression. These are germinal centre B-cells. The blue arrow demonstrates how mature B-cells gain and then lose CD10 expression as they pass through the germinal centre and then exit again.

### iii) Presence of clonal populations

In the vast majority of presenting B-cell neoplasms there will be a large population of monoclonal cells expressing a single kappa or lambda light chain. Light chain expression is straight forward to assess using flow cytometry once circulating immunoglobulin has been removed by washing. A monoclonal population will result in perturbation of the balance of cells expressing kappa and lambda light chains, resulting in a kappa:lambda ratio outside the reference range. Examples of monoclonal populations can be seen in Figure 7. Light chain restriction, however, is

not always indicative of clonality and there are examples in the literature of non-clonal reactive B-cell populations which exhibit light chain restriction (<sup>9</sup>).

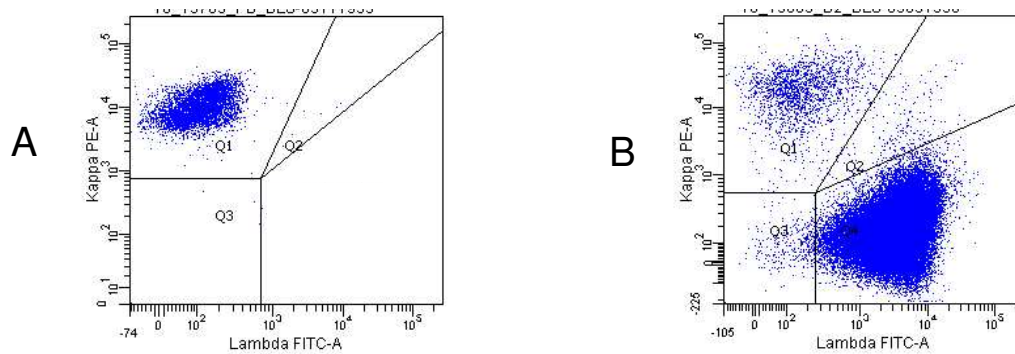


Figure 7. Monoclonal populations in samples from presentation lymphoma cases

The sample represented in Plot A has a kappa positive neoplasm and no normal B-cells remain. In Plot B, although there are some normal polyclonal cells still present, there is still an obvious lambda positive population.

Improvements in the sensitivity and specificity of flow cytometric assays have made it possible to detect small populations of neoplastic cells. In samples taken following treatment or in staging marrow aspirates the neoplastic cells may be mixed with normal progenitor and mature polyclonal B-cells. In these instances reliance on assessment of the kappa:lambda ratio is not sufficient as there may not be a large enough number of abnormal cells present to take the ratio outside the normal range. An example of an occult monoclonal population can be seen in Figure 8. In these cases it is essential to have detailed knowledge about the immunophenotype of the neoplastic cells so they can be distinguished from normal B-cells without the use of clonality.

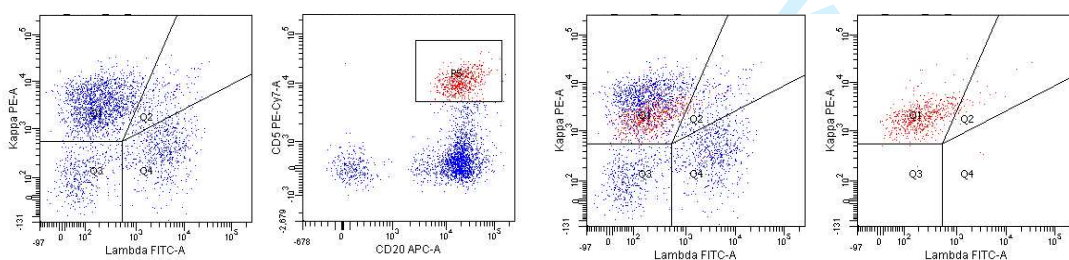


Figure 8. Small monoclonal populations can be hidden in a normal polyclonal background. The series of plots above shows the gated B-cells from a BM sample. The kappa:lambda ratio is normal (1.9:1) but further analysis shows a small proportion of the B-cells are CD5+. The final two plots show the CD5+ B-cells in red and the population is monoclonal (Kappa+).

#### iv) Detailed immunophenotype

Once a population of interest has been successfully gated it is possible to examine the immunophenotype of those cells in detail. Each sub-population of normal cells has a specific immunophenotype and abnormal phenotypes characterise many of common disease entities. If a disease-specific phenotype has been identified then it is possible to distinguish these neoplastic cells from all other populations based on differences in antigen expression in both presentation and follow up specimens (<sup>10</sup>).

For some antigens, these differences will be positivity compared to negativity. For others, it is intensity of expression that is altered and the quantitative nature of flow cytometry data can be a real advantage over other phenotyping techniques where intensity is much harder to assess.

A detailed immunophenotype is not only diagnostically important but also generates information which can be prognostically important. Expression of certain antigens, for example CD38 in CLL (<sup>11</sup>), have prognostic implications. Antigens within the extended phenotype will also influence decisions regarding treatment with antibody-based regimens such as Alemtuzamab (anti-CD52) or Rituximab (anti-CD20). Treatment itself can affect the level of expression of certain markers, for example B-cells in a patient that has been treated with Rituximab will show significantly weaker CD20 expression than prior to treatment (<sup>12</sup>). This is important to consider in the construction of panels that may be used post-treatment. Any markers to be used in an MRD setting, especially those that will be relied upon for gating, should ideally be 'therapy proof'.

#### Flow cytometric characteristics of individual B-cell malignancies

The immunophenotypes of some of the most commonly seen mature B-cell neoplasms can be seen in Table 1. CLL is perhaps the most immunophenotypically well-defined of all the mature B-cell lymphoproliferative disorders. Due to the high levels of circulating disease present in most cases, access to material for flow cytometric analysis is straight-forward and in many instances a diagnosis can be made on the basis of a peripheral blood sample alone. The immunophenotype of a typical case of CLL can be seen in Figure 9. There are also a number of antigens which have been identified as prognostic factors in CLL, including CD38, ZAP70 and CD49d (<sup>11, 13, 14</sup>).

Disease entity	Typical phenotype	Atypical expression
Chronic Lymphocytic Leukaemia (CLL)	CD19+, CD20+(weak), CD5+, CD81+(weak), CD79b+(weak), CD43++, CD23+, CD200+, CD52++, CD10-, CD38variable, weak surface immunoglobulins such as kappa/lambda, IgM and IgD	Atypical cases can show weak or absent CD5 expression, lack of CD23, strong CD20 or combinations of the aforementioned.
Hairy Cell Leukaemia (HCL)	CD19++, CD20++, very strong surface immunoglobulin, CD22++, CD103+, CD25+, CD11c+, CD10-, CD5-	Atypical cases can lack CD25 expression and are classified as variant HCL (vHCL). CD10 positivity can be seen in a significant number of individuals, with reported frequencies ranging from 10% to 26% of cases ( <sup>18, 19, 20</sup> ).
Mantle cell lymphoma (MCL)	CD19+, CD20+, CD5+, CD23-, CD200-, CD52++, CD10-	Atypical cases can be CD5 negative and instances with CD23 and/or CD200 expression are not uncommon
Follicular Lymphoma (FL)	CD19+(wk), CD20+, CD10+, CD38+, CD43-	Atypical cases, reported as approximately 50% of samples( <sup>10</sup> ), can have weak or absent CD10 expression and the majority of these cases are high-grade ( <sup>24</sup> ).

Table 1. Examples of immunophenotypes for some of the most commonly seen mature B-cell neoplasms.



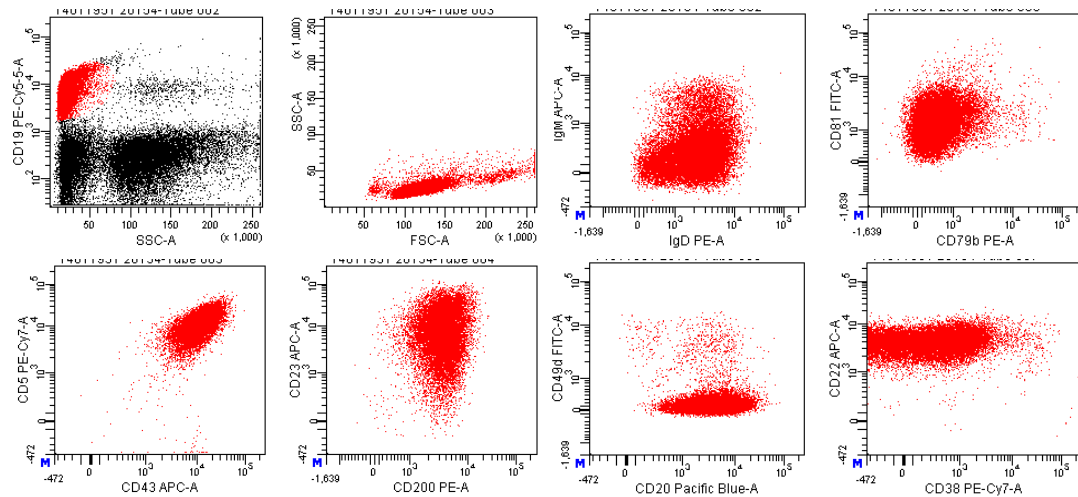


Figure 9. Typical immunophenotype of CLL.

The plots show a BMA sample from a patient at presentation. B-cells are gated using CD19 and the subsequent plots show that the cells have a typical CLL phenotype.

As treatment regimens for CLL have become more effective it has become more important to be able to detect minimal residual disease (MRD) following therapy. It has been shown that presence of MRD predicts a poorer outcome<sup>(15)</sup> and that flow cytometry can be more effective in this setting than conventional PCR<sup>(16)</sup>. There is now an internationally standardised approach to assessing MRD by flow and the assay can detect CLL cells when they represent as few as 0.01% of total leucocytes<sup>(17)</sup>. Figure 10 demonstrates how the different B-cell populations present in a post-treatment bone marrow sample can be discriminated from each other.

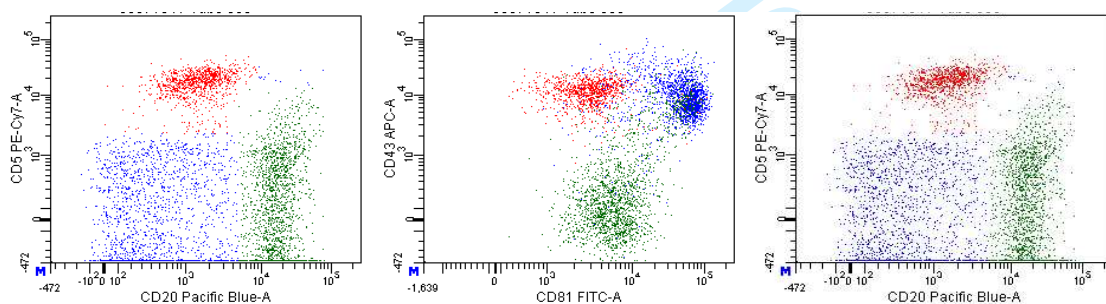


Figure 10. MRD analysis in CLL

The plots above show analysis of a bone marrow with a minimal CLL population and demonstrate how the use of a few carefully selected antigens can discriminate between CLL cells (red), normal mature B-cells (green) and B-cell progenitors (blue). B-cells have been gated in a CD19 vs SSC plot and are 1.6% of total leucocytes. CLL cells represent 0.32% of total leucocytes.

Certain markers are extremely specific for a diagnostic entity. Expression of CD103 is almost unique to cases of HCL and vHCL, with the only exceptions being rare cases of atypical splenic marginal zone lymphoma (<sup>19</sup>). HCL is immunophenotypically well defined, as seen in Figure 11, and this specific phenotype has enabled development of successful MRD assays (<sup>21</sup>). Unfortunately, markers with the specificity of CD103 are rare and the majority of diagnoses rely on complex composite immunophenotypes.

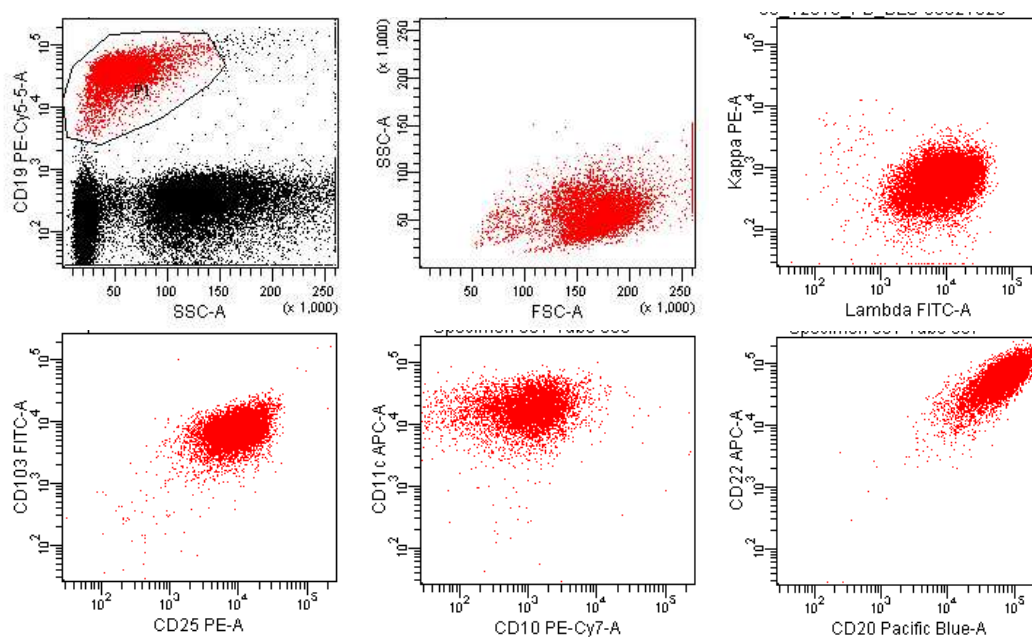


Figure 11. Immunophenotype of typical HCL

The above plots show analysis of a peripheral blood sample from a patient with a large population of neoplastic B-cells. These have a typical phenotype for HCL with strong CD103, CD25, CD11c and CD22 expression; note the larger than usual SSC and FSC which is caused by the projections on the cell surface rather than the size of the cell.

Flow cytometry plays a crucial role in directing FISH or molecular testing, especially for CCND1/IGH@ or t(11;14) in mantle cell lymphoma. Particularly in peripheral blood samples, it is critical that atypical cases are not mis-classified as CLL or as a CD5-negative BLPD without proceeding to molecular analysis. Recently, CD200 has been identified as an antigen that can be used to distinguish between MCL and CLL. Although atypical cases exist, in general the neoplastic cells in MCL are CD200 negative and those in CLL are CD200 positive (<sup>23</sup>), as shown in Figure 12.



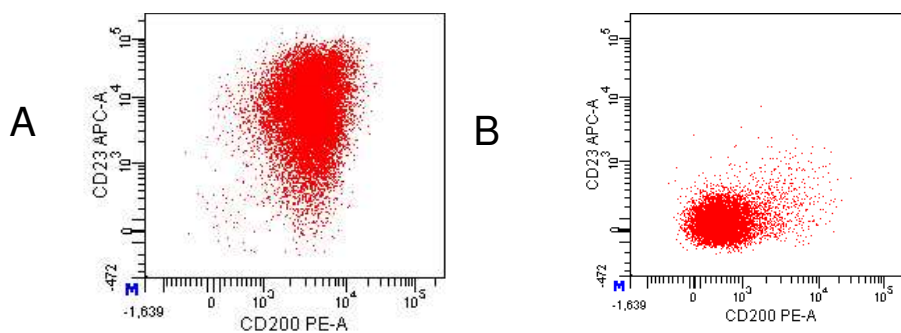


Figure 12. Use of CD200 to discriminate CLL from MCL

Plot A shows gated B-cells from a BMA sample involved with CLL. The cells demonstrate expression of CD23 and CD200. Plot B displays B-cells from a patient with MCL. The cells have a typical MCL phenotype and are negative for both markers. CD200 is extremely useful in cases of MCL which exhibit atypical CD23 expression.

As well as analysing antigens on the cell surface, it is also possible to detect intracellular proteins by flow cytometry. BCL2 expression can distinguish follicular lymphoma cells from other CD10+ events, namely normal progenitors and non-malignant germinal centre cells<sup>(25)</sup>. It is therefore useful for differentiating between case of neoplasia and reactive hyperplasia<sup>(26)</sup>. An example is depicted in Figure 13.

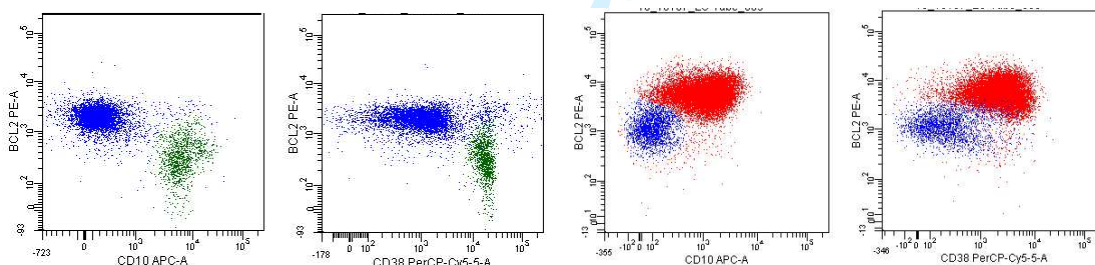


Figure 13. Immunophenotyping of follicular lymphoma

BCL2 expression can be used to distinguish between normal mature B-cells (shown in blue), B-cell progenitors (in green) and follicular lymphoma cells (in red).

Some other B-cell lymphomas still remain challenging for flow cytometrists, particularly where there is no single disease specific phenotype which characterises the entity and there is great heterogeneity between cases, This is especially the case for diffuse large B-cell lymphoma (DLBCL). In the last few years, gene expression profiling has generated large amounts of data about the neoplastic cells of many haematological malignancies, some of which has been applied to improve diagnostic

assays for mature lymphoid neoplasms (<sup>27</sup>). Protein expression profiling is a novel method for identification of disease-specific antigens that has been used successfully in the development of new combinations of markers to detect MRD in CLL (<sup>28</sup>). As new candidate markers emerge, disease-specific phenotypes for the more heterogeneous lymphomas should follow.

In addition to heterogeneity of antigen expression, flow cytometric analysis of large cell lymphomas, such as Burkitt lymphoma (BL) and DLBCL, present several specific technical difficulties. The neoplastic cells in these disease entities are prone to high levels of apoptosis and cells can be fragile or necrotic making interpretation of flow cytometry data difficult (<sup>29</sup>). If the sample is viable, the large size of the neoplastic cells can place them far outside a normal lymphocyte SSC/FSC gate. This can mean the relevant cells are excluded from analysis completely if lymphocytes are gated too tightly. Many of the large cell lymphomas can also show down-regulation of CD19 and/or CD20 expression (<sup>8</sup>) which can make gating of the neoplastic population difficult, as previously discussed in the section on identification of cell lineage.

Distinguishing between lymphoplasmacytic lymphoma (LPL) and the various types of marginal zone lymphoma (MZL) can be difficult. The malignant population of LPL is composed of cells at a variety of different stages of differentiation, including lymphocytes, plasmacytoid lymphocytes and terminally differentiated plasma cells. Several other lymphoma entities, such as MZL, can involve lymphocytes that show plasmacytic differentiation but have no true plasma cells present. Plasma cell immunophenotyping has been shown to be helpful in distinguishing LPL from other types of lymphoma by identifying those cases which have true plasma cell populations as compared to those which have plasmacytoid lymphocytes (<sup>30</sup>).

Hodgkin lymphoma (HL) differs greatly from other types of B-cell lymphoma. The neoplastic cells in HL, the Hodgkin and Reed-Sternburg (HRS) cells, are usually only present in small numbers and often show lack of classical lineage-specific markers such as CD19 and CD20. This means the normal approaches for identification and classification of malignant cells cannot be applied. Until recently, the use of flow cytometry in HL has been limited to evaluation of the reactive T-cell populations which are associated with the HRS cells (<sup>31</sup>). However, a novel assay using 10 colour

flow cytometry was published in 2009 (<sup>32</sup>) which allowed identification and quantification of HRS cells with high sensitivity and specificity. As the number of colours available to routine laboratories increases, immunophenotyping of HRS cells may become more generally adopted.

### ***Flow cytometric approach to the classification of T-cell and NK-cell neoplasms.***

The analysis of suspected T-cell and NK cell derived tumour follows the same steps which have been described above for B-cell malignancies.

#### **i) Cell lineage**

There are numerous antigens which indicate T-cell (CD2, CD3, CD5 and CD7) or NK-cell (CD2, CD16, CD56) lineage. Surface expression of CD3 occurs during T-cell maturation, with lymphoblasts showing only cytoplasmic CD3 positivity. CD2, CD5 and CD7 expression can be seen at all stages of development, although minor sub-populations which lack expression may exist in samples from healthy individuals. Unfortunately, the markers that are commonly relied upon for identification of T-cell lineage in normal cells often show weak or completely absent expression in neoplastic populations. As with the B-cell neoplasms, utilisation of multiple antigens for gating can improve identification but populations which are negative for several T-cell-lineage-related antigens will still pose a problem and are most likely to be misclassified.

#### **ii) Developmental stage**

Mature T-cells are defined by the presence of surface CD3 expression. T-cell lymphoblasts are negative for surface CD3 and positive for cytoplasmic CD3 and also express CD1a. Following TCR gene rearrangement, the cells become surface CD3 positive and exit from the thymus into the peripheral circulation as naïve T-cells. There are a vast number of mature T-cell sub-populations which can be present in a normal sample, including regulatory, effector and memory populations. Mature T-cells are often split crudely into helper (CD4+) and cytotoxic (CD8+) components and large variations in the ratio of these populations can be seen in normal and reactive

populations. Although not definitive of the presence of a malignant population, skewing of the CD4;CD8 ratio warrants further investigation.

### **iii) Presence of clonal populations**

Presence of a clonal T-cell population results in expansion of a group of cells with a restricted phenotype. TCR repertoire can be assessed by flow cytometry using antibodies which recognise products of V and D domains following VDJ recombination. The most commonly used is V- $\beta$  analysis and, although the gold standard for this analysis is PCR, flow cytometric evaluation has been shown to be an efficient substitute in both peripheral blood and lymph node specimens (<sup>33, 34</sup>). In a normal sample there is heterogeneous expression of V- $\beta$  subtypes, resulting in a population of T-cells expressing a large variety of V- $\beta$  family members. A clonal T-cell population will have a far more homogeneous pattern of expression. As with light chain restriction in B-cell populations, non-malignant reactive populations can appear clonal and oligoclonality is seen in healthy individuals as age increases (<sup>35</sup>). Samples which contain small numbers of T-cells, such as those from aplastic patients, can falsely appear to be clonally restricted. Clonal populations can also be hidden amongst a background of normal polyclonal cells so results must be interpreted carefully.

### **iv) Detailed immunophenotype**

The large number of normal sub-populations present in the T-cell and NK cell lineages makes identification of phenotypically abnormal population more challenging than for the B-cell lineage. Even so, there are disease specific phenotypes established for some disease entities and these can be used to classify abnormal populations.

Treatment with therapeutic antibodies is also a possibility for some T-cell neoplasms and assessment of expression of CD52 (which is needed for treatment with Alemtuzamab) and CD25 (against which Daclizumab is directed) is an important part of the extended phenotyping process.

Flow cytometric characteristics of individual T-cell malignancies

The situation described for the B-cell neoplasms is similar in the T-cell lineage disorders. There are a proportion of entities which are immunophenotypically well-

defined and these are shown in Table 2. These entities tend to be those with high levels of circulating disease which means that large numbers of cells are usually available for analysis in a peripheral blood sample. Examples of flow cytometry plots for T-cell Prolymphocytic Leukaemia (T-PLL), Large Granular Lymphocyte Leukaemia (LGL) and Sezary syndrome (SS) can be seen in Figures 14, 15 and 16 respectively. Again, a well-defined phenotype can be used to develop a successful MRD assay.

Disease entity	Typical phenotype	Atypical expression
T-cell Prolymphocytic Leukaemia (T-PLL)	CD3+, CD2+, CD7+, CD5+, CD1a-, CD52++, CD4+, CD8-	Atypical cases can show a distinct populations which co-expresses CD4 and CD8, a characteristic not seen in other T-cell malignancies. There are also rare case which are CD8+, CD4-
Large Granular Lymphocyte Leukaemia (LGL)	CD3+, CD8+, TCR $\alpha\beta$ , CD5wk/-, CD7wk/-, CD57+, CD16+	Atypical cases include examples which are CD4+, TCR $\alpha\beta$ + and also CD8+, TCR $\gamma\delta$ expressing variants.
Sezary syndrome (SS)	CD3+, CD2+, CD5+, CD4+, CD7-, CD26-, TCR $\alpha\beta$	Atypical cases include down-regulation of T-cell antigens other than CD7, most commonly CD2 and also rare examples with CD8 expression. Until recently, lack of CD26 expression was thought to be a feature of all Sezary cells but current evidence suggests that only 60% of SS cases show CD26 negativity and that expression is also lacking in a third of mycosis fungoides cases ( <sup>36</sup> ).

Table 2. Examples of immunophenotypes for some of the most commonly seen mature T-cell neoplasms.

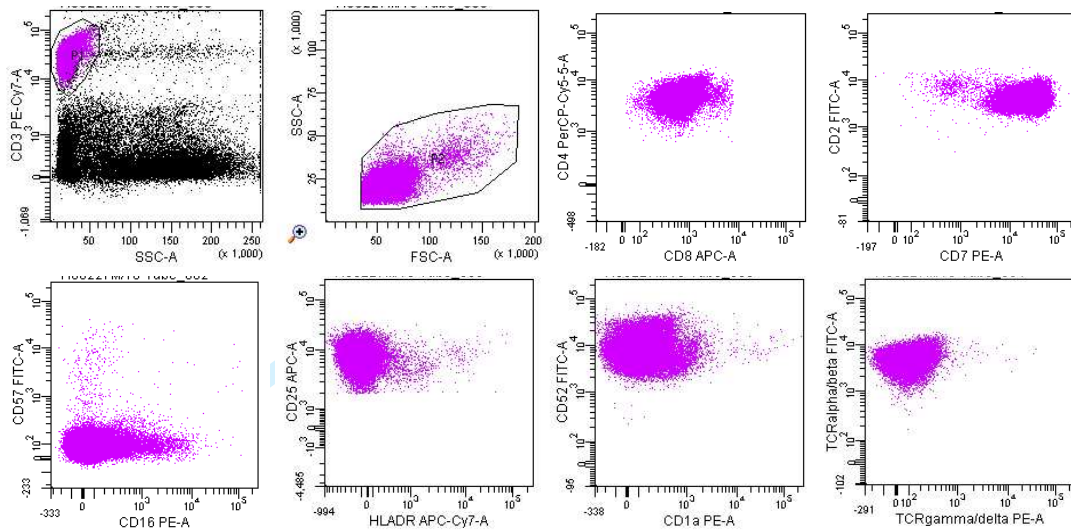


Figure 14. Typical phenotype of T-PLL.

T-cells have been gated using CD3 vs SSC and then a secondary gate (not shown) has been used to select the T-PLL cells based on CD4 positivity.

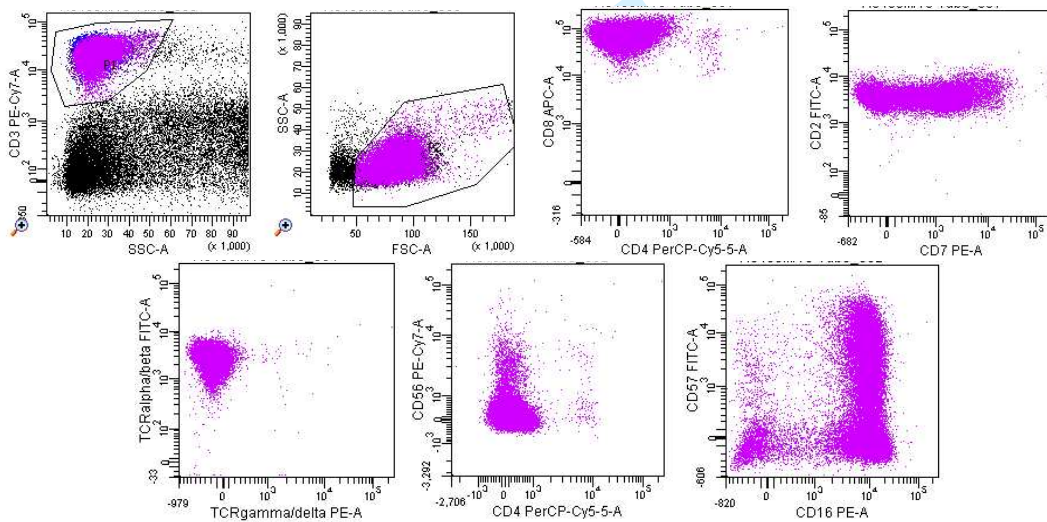


Figure 15. Typical phenotype of T-cell large granular lymphocytic leukaemia  
Cells gated using CD3 are CD8+, CD4-, TCR $\alpha\beta$ +, CD57+ and CD16+.



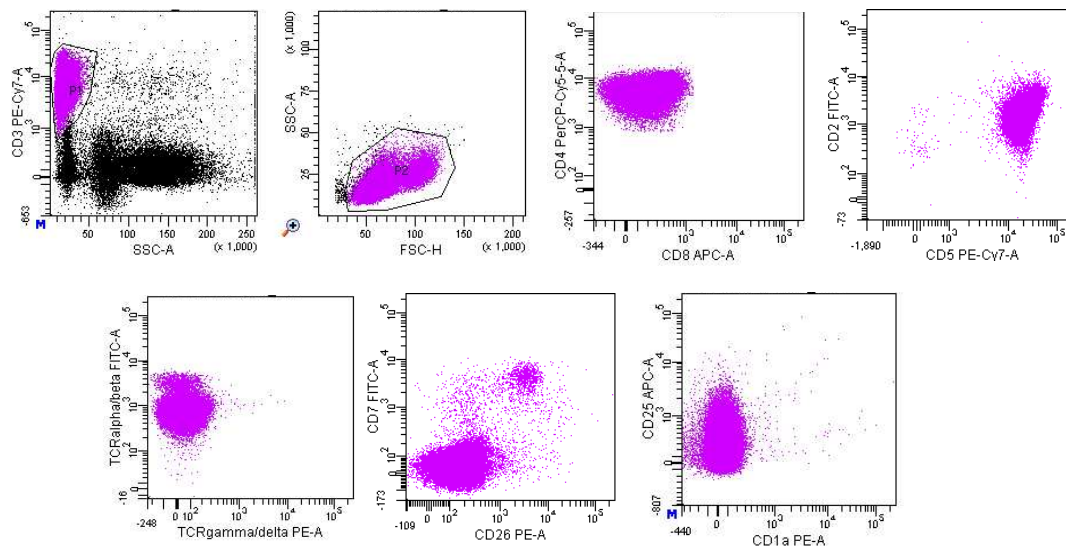


Figure 16. Typical phenotype of the neoplastic cells in Sezary syndrome  
CD3+ gated cells show the characteristic CD4+, CD8-, CD2+, CD7-, CD26- phenotype

The diagnosis and classification of other types of T-cell malignancy remains problematic. The rarity of these cases makes it extremely difficult to create immunophenotypic databases, so there is a lack of knowledge regarding antigens which may be useful in distinguishing between the different disease entities. The entities themselves are in many cases poorly defined, with large amounts of heterogeneity within the categories. The classification of T-cell lymphomas is not as established as that of the B-cell neoplasms and many are classified as peripheral T-cell lymphoma, not otherwise specified.

There are a number of specific technical problems which has inhibited the development of flow cytometric methods for the diagnosis and classification of T-cell malignancies. The typical sites of presentation of T-cell lymphomas is more variable than for B-cell lymphomas with a higher proportion originating at extranodal sites. This can generate sampling issues and reduce the number of unfixed specimens received that are suitable for flow cytometric analysis. Down-regulation of T-cell markers is common in T-cell malignancies and this can make lineage identification difficult, with cells having a “null” phenotype<sup>(37)</sup>. In other cases, for example Anaplastic Large Cell Lymphoma (ALCL), the neoplastic cells are large and can be low in number, meaning adaptation of gating strategies is necessary<sup>(38)</sup>.

## Advantages and disadvantages of flow cytometry as a technique

Analysis of flow cytometric data is an objective process involving both qualitative and quantitative measurements. Results are not influenced by the same levels of subjectivity as are present in morphological evaluation. Cells are defined by their pattern of antigen expression and in most cases lineage identification is straight forward. The multiparametric nature of flow cytometry data allows numerous antigens to be assessed simultaneously, making it possible to examine antigen expression and co-expression on specific populations. Large numbers of cells can be analysed very rapidly, with most laboratories routinely collecting data on a minimum of 100,000 cells at presentation and between 500,000 and 1,000,000 cells in an MRD setting. Results can be delivered more quickly than histological analysis as samples do not need to be subjected to the lengthy fixing and processing stages necessary for cytomorphological and immunohistochemical analysis.

The data generated by flow cytometry is only as reliable as the sample from which it results. For tissue biopsies, especially those which are core or fine-needle in origin, and bone marrow aspirates, lack of neoplastic cells in the sample does not necessarily constitute absence of disease<sup>(39)</sup>. Many neoplastic nodes or masses can show lack of uniform involvement and inaccurate sampling can lead to a biopsy which is devoid of malignant cells but also unrepresentative of the total mass. Accuracy can be improved by performing biopsies with CT guidance but it is still important to consider that a normal result on a minimally sized biopsy does not exclude a diagnosis of lymphoma. In bone marrow aspirate samples, lymphoma cells can aggregate in clusters which adhere to the trabeculae and are therefore not sampled when an aspirate is taken. The quality of the aspirate sample can also be affected by dilution of the collection with peripheral blood. In both these cases the samples will not be representative of the situation *in vivo*.

Increasingly, centres are moving away from full excisional biopsies of lymph nodes, favouring instead less invasive procedures such as fine needle aspiration (FNA) or needle core biopsy<sup>(40)</sup>. Although these procedures are favourable for the patient in terms of discomfort and recovery time, the resulting sample offers far fewer cells and greatly increases the diagnostic challenges. If the paucity of the sample is such that a



diagnosis cannot be reached then a surgical biopsy will be inevitable causing a delay in treatment which may, in some cases, affect patient outcome. Flow cytometric data can greatly assist in reaching a diagnostic conclusion in these cases (<sup>41</sup>, <sup>42</sup>) with studies showing the sensitivity of flow cytometry in the context of FNAs been equal to or greater than that of PCR (<sup>43</sup>). Knowledge regarding cellular composition of the sample, presence of monoclonal populations and presence of phenotypic abnormalities will facilitate the most efficient use of the limited cells or tissue samples which are available by directing further testing. The integration of flow cytometric analysis of cellular aspirate and histological evaluation of a needle-core biopsy is a promising approach to improving the applicability of minimally invasive diagnostic techniques.

The need for fresh tissue or cells restricts the number of biopsies that can be analysed by flow cytometry. In many centres it is not routine practice to acquire unfixed tissue even when lymphoma is suspected clinically. This is despite the advantages of having access to flow cytometric data, improved molecular analysis and, in some cases, improved fixation and processing of routine histological blocks. Hopefully, this will be improved by the centralisation of diagnostic services for haematological malignancies which will provide wider access to the full range of diagnostic techniques.

Improvements in the sensitivity and specificity of flow cytometric assays have made it possible to detect small populations of neoplastic cells in a background of normal mature and progenitor cells. Several groups have shown the presence of occult monoclonal B-cell populations in peripheral blood samples from normal individuals (<sup>44</sup>, <sup>45</sup>). Flow cytometric analysis has also been shown to be more sensitive than histological assessment in the evaluation of bone marrow samples referred for staging of a known mature B-cell neoplasm (<sup>16</sup>, <sup>46</sup>). The clinical implications of finding these small clonal populations are still unclear. The presence of bone marrow involvement elevates the disease stage that is assigned to the patient and this has a direct impact on treatment decisions. Further work needs to be carried out to assess the impact which very low levels of bone involvement have on patient outcome, so that new guidelines can be developed for staging in these situations. The clinical significance of the presence of occult monoclonal populations, which are increasingly detected in healthy

individuals, requires further clarification but recent data suggest that the rate of progression may be relatively low. (<sup>47</sup>, <sup>48</sup>). As flow cytometric technology continues to improve, it will become possible to find smaller populations of cells in all types of samples. It is critical that these results are considered in the clinical context to derive the maximum value from them and to ensure the best care for the patient, acknowledging that no intervention may be required in many of these cases.

### **The future of flow cytometry in the analysis of mature lymphoid malignancies**

The future of flow cytometry will undoubtedly bring cytometers with a greater numbers of lasers and parameters and this will be accompanied by new choices of fluorochromes. As our knowledge of the haematopoietic system and the cells within it continue to increase, there will also be new antigens to be investigated for their utility in the development of more disease-specific phenotypes. The vast majority of antibodies currently available are directed against antigens expressed on the cell surface but as techniques improve there will be an increase in reagents to examine the internal proteins.

As the complexity of flow cytometry increases, it will become more important for diagnostic and MRD approaches to be standardised. The Bethesda Consensus (<sup>4</sup>, <sup>49</sup>) produced recommendations on the use of reagents and the reporting of immunophenotyping results but there is a growing need for standardisation of antibody panels. The Euroflow Consortium is a group of diagnostic laboratories and biotechnology companies from across Europe (<sup>50</sup>). The Euroflow project aims to develop specific flow cytometric tests for the diagnosis and monitoring of haematological malignancies. A standardised method for testing of potentially neoplastic samples will increase reliability and reproducibility of diagnostic assays. Flow cytometric acquisition generates large amounts of data that needs to be analysed by highly experienced operators. These individuals are trained to recognise subtle changes in antigen expression to identify abnormal cell populations (<sup>49</sup>). Infinicyt (Cytognos, Salamanca) is a newly developed software program for flow cytometric analysis which has been created as part of the Euroflow project and approaches evaluation of data in a completely new way (<sup>51</sup>). One of the aims of this development

is a software program which will allow automated analysis of data by comparing test data with a database of normal and malignant samples (<sup>52</sup>). This would allow high levels of reproducibility in flow cytometry analysis.

Recently of a new flow technology has been introduced, (ImageStream Amnis Corporation, Seattle), which combines flow cytometric fluorescence measurements with digital images. This allows simultaneous assessment of surface antigens and morphology (<sup>53</sup>). This multispectral imaging technique has been successfully used to assess cells of the haematopoietic system (<sup>54</sup>) and offers interesting possibilities for the investigation of mature lymphoid malignancies.

### Concluding remarks

Over the last decade, flow cytometry has been established as a critical tool in the diagnosis of haematological malignancies. This review has summarised the contribution the technique currently makes to the diagnosis and management of mature lymphoid neoplasms. Recent improvements in technology are beginning to yield assays with much higher levels of disease specificity and sensitivity which can be applied in routine diagnostic laboratories. Many types of lymphoma can now be correctly classified by flow cytometric analysis independent of other diagnostic modalities which allows much more robust integrated diagnostic protocols to be developed. The development of highly sensitive techniques will play an increasing role in disease monitoring through and beyond treatment. Flow cytometry now has the potential to improve substantially the diagnosis and monitoring of patients with lymphoma. To realise this potential fully will require a change in the way specimens are acquired and in the organisation of specialist haematopathology service.

### References

1. Jaffe, E.S., Harris, N.L., Stein, H. & Isaacson, P.G. Classification of lymphoid neoplasms: the microscope as a tool for disease discovery. *Blood* **112**, 4384-4399 (2008).
2. Jaffe, E., Harris, N. & Stein, H. *Tumours of the Haematopoietic and Lymphoid*

- Tissues. World Health Organisation Classification of Tumours.* (International Agency for Research on Cancer: Lyon, France, 2001).
3. Swerdlow, S., Campo, E., Harris, N. & et al *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.* (International Agency for Research on Cancer: Lyon, France, 2008).
  4. Wood, B.L. et al. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: optimal reagents and reporting for the flow cytometric diagnosis of hematopoietic neoplasia. *Cytometry B Clin Cytom* **72 Suppl 1**, S14-22 (2007).
  5. McMichael, A.J. et al. A human thymocyte antigen defined by a hybrid myeloma monoclonal antibody. *Eur. J. Immunol* **9**, 205-210 (1979).
  6. Wood, B. 9-color and 10-color flow cytometry in the clinical laboratory. *Arch. Pathol. Lab. Med* **130**, 680-690 (2006).
  7. Nadler, L.M. et al. A unique cell surface antigen identifying lymphoid malignancies of B cell origin. *J. Clin. Invest* **67**, 134-140 (1981).
  8. Yang, W. et al. Diminished Expression of CD19 in B-cell Lymphomas. *Cytometry. Part B, Clinical Cytometry* **63B**, 28-35 (2005).
  9. Kroft, S.H. Monoclones, monotypes, and neoplasia pitfalls in lymphoma diagnosis. *Am. J. Clin. Pathol* **121**, 457-459 (2004).
  10. Morice, W.G. et al. Predictive value of blood and bone marrow flow cytometry in B-cell lymphoma classification: comparative analysis of flow cytometry and tissue biopsy in 252 patients. *Mayo Clin. Proc* **83**, 776-785 (2008).
  11. Matutes, E. New additions to antibody panels in the characterisation of chronic lymphoproliferative disorders. *Journal of Clinical Pathology* **55**, 180-183
  12. Hiraga, J. et al. Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance. *Blood* **113**, 4885-4893 (2009).
  13. Trojani, A. et al. ZAP-70, IgVh, and cytogenetics for assessing prognosis in chronic lymphocytic leukemia. *Cancer Biomark* **6**, 1-9 (2010).
  14. Gattei, V. et al. Relevance of CD49d protein expression as overall survival and progressive disease prognosticator in chronic lymphocytic leukemia. *Blood* **111**, 865-873 (2008).
  15. Moreton, P. et al. Eradication of minimal residual disease in B-cell chronic lymphocytic leukemia after alemtuzumab therapy is associated with prolonged

- survival. *J. Clin. Oncol* **23**, 2971-2979 (2005).
16. Talaulikar, D. et al. Clinical role of flow cytometry in redefining bone marrow involvement in diffuse large B-cell lymphoma (DLBCL) - a new perspective. *Histopathology* **52**, 340-347 (2008).
  17. Rawstron, A.C. et al. International standardized approach for flow cytometric residual disease monitoring in chronic lymphocytic leukaemia. *Leukemia* **21**, 956-964 (2007).
  18. Jasionowski, T.M., Hartung, L., Greenwood, J.H., Perkins, S.L. & Bahler, D.W. Analysis of CD10+ hairy cell leukemia. *Am. J. Clin. Pathol* **120**, 228-235 (2003).
  19. Dong, H.Y., Weisberger, J., Liu, Z. & Tugulea, S. Immunophenotypic analysis of CD103+ B-lymphoproliferative disorders: hairy cell leukemia and its mimics. *Am. J. Clin. Pathol* **131**, 586-595 (2009).
  20. Robbins, B.A. et al. Diagnostic application of two-color flow cytometry in 161 cases of hairy cell leukemia. *Blood* **82**, 1277-1287 (1993).
  21. Sausville, J.E. et al. Minimal residual disease detection in hairy cell leukemia. Comparison of flow cytometric immunophenotyping with clonal analysis using consensus primer polymerase chain reaction for the heavy chain gene. *Am. J. Clin. Pathol* **119**, 213-217 (2003).
  22. Swerdlow, S.H. & Williams, M.E. From centrocytic to mantle cell lymphoma: a clinicopathologic and molecular review of 3 decades. *Hum. Pathol* **33**, 7-20 (2002).
  23. Palumbo, G.A. et al. CD200 expression may help in differential diagnosis between mantle cell lymphoma and B-cell chronic lymphocytic leukemia. *Leuk. Res* **33**, 1212-1216 (2009).
  24. Xu, Y., McKenna, R.M. & Kroft, S.H. Assessment of CD10 in the Diagnosis of Small B-cell Lymphomas. *American* **117**, 291-300 (2002).
  25. Cook, J.R., Craig, F.E. & Swerdlow, S.H. bcl-2 Expression by Multicolor Flow Cytometric Analysis Assists in the Diagnosis of Follicular Lymphoma in Lymph Node and Bone Marrow. *American Journal of Clinical Pathology* **119**, 145-151 (2003).
  26. Laane, E. et al. Flow Cytometric Immunophenotyping Including Bcl-2 Detection on Fine Needle Aspirates in the Diagnosis of Reactive Lymphadenopathy and Non-Hodgkin's Lymphoma. *Cytometry. Part B, Clinical Cytometry* **64B**, 34-42 (2005).

27. Dunphy, C.H. Gene expression profiling data in lymphoma and leukemia: review of the literature and extrapolation of pertinent clinical applications. *Arch. Pathol. Lab. Med* **130**, 483-520 (2006).
28. Rawstron, A.C., de Tute, R., Jack, A.S. & Hillmen, P. Flow cytometric protein expression profiling as a systematic approach for developing disease-specific assays: identification of a chronic lymphocytic leukaemia-specific assay for use in rituximab-containing regimens. *Leukemia* **20**, 2102-2110 (2006).
29. Bertram, H.C., Check, I.J. & Milano, M.A. Immunophenotyping Large B-Cell Lymphomas. *American Journal of Clinical Pathology* **116**, 191-203 (2001).
30. Morice, W.G., Chen, D., Kurtin, P.J., Hanson, C.A. & McPhail, E.D. Novel immunophenotypic features of marrow lymphoplasmacytic lymphoma and correlation with Waldenström's macroglobulinemia. *Mod. Pathol* **22**, 807-816 (2009).
31. Hudnall, S.D., Betancourt, E., Barnhart, E. & Patel, J. Comparative Flow Immunophenotypic Features of the Inflammatory Infiltrates of Hodgkin Lymphoma and Lymphoid Hyperplasia. *Cytometry. Part B, Clinical Cytometry* **74B**, 1-8 (2008).
32. Fromm, J.R., Thomas, A. & Wood, B.L. Flow cytometry can diagnose classical hodgkin lymphoma in lymph nodes with high sensitivity and specificity. *Am. J. Clin. Pathol* **131**, 322-332 (2009).
33. van den Beemd, R. et al. Flow cytometric analysis of the Vbeta repertoire in healthy controls. *Cytometry* **40**, 336-345 (2000).
34. Salameire, D. et al. Efficient characterization of the TCR repertoire in lymph nodes by flow cytometry. *Cytometry A* **75**, 743-751 (2009).
35. Giachino, C. et al. Clonal expansions of V delta 1+ and V delta 2+ cells increase with age and limit the repertoire of human gamma delta T cells. *Eur. J. Immunol* **24**, 1914-1918 (1994).
36. Kelemen, K., Guitart, J., Kuzel, T.M., Goolsby, C.L. & Peterson, L.C. The usefulness of CD26 in flow cytometric analysis of peripheral blood in Sézary syndrome. *Am. J. Clin. Pathol* **129**, 146-156 (2008).
37. Kinney, M.C. & Jones, D. Cutaneous T-cell and NK-cell lymphomas: the WHO-EORTC classification and the increasing recognition of specialized tumor types. *Am. J. Clin. Pathol* **127**, 670-686 (2007).
38. Muzzafar, T., Wei, E.X., Lin, P., Medeiros, L.J. & Jorgensen, J.L. Flow

- cytometric immunophenotyping of anaplastic large cell lymphoma. *Arch. Pathol. Lab. Med* **133**, 49-56 (2009).
39. Dunphy, C.H. Contribution of Flow Cytometric Immunophenotyping to the Evaluation of Tissues With Suspected Lymphoma? *Cytometry* **42**, 296-306 (2000).
  40. Sandhaus, L.M. Fine-Needle Aspiration Cytology in the Diagnosis of Lymphoma. *Am* **113**, 623-627 (2000).
  41. Gong, J.Z., Williams, D.C., Liu, K. & Jones, C. Fine-Needle Aspiration in Non-Hodgkin Lymphoma. *American Journal of Clinical Pathology* **117**, 880-888 (2002).
  42. Meda, B.A. et al. Diagnosis and Subclassification of Primary and Recurrent Lymphoma. *American Journal of Clinical Pathology* **113**, 688-699
  43. Maroto, A., Martinez, M., Martinez, M.A., de Agustin, P. & Rodriguez-Peralto, J.L. Comparative analysis of immunoglobulin polymerase chain reaction and flow cytometry in fine needle aspiration biopsy differential diagnosis of non-Hodgkin B-cell lymphoid malignancies. *Diagn. Cytopathol* **37**, 647-653 (2009).
  44. Rawstron, A.C. et al. Monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia. *N. Engl. J. Med* **359**, 575-583 (2008).
  45. Nieto, W.G. et al. Increased frequency (12%) of circulating chronic lymphocytic leukemia-like B-cell clones in healthy subjects using a highly sensitive multicolor flow cytometry approach. *Blood* **114**, 33-37 (2009).
  46. Talaulikar, D. & Dahlstrom, J.E. Staging bone marrow in diffuse large B-cell lymphoma: the role of ancillary investigations. *Pathology* **41**, 214-222 (2009).
  47. Dagklis, A., Fazi, C., Scarfo, L., Apollonio, B. & Ghia, P. Monoclonal B lymphocytosis in the general population. *Leuk. Lymphoma* **50**, 490-492 (2009).
  48. Rawstron, A.C. & Hillmen, P. Clinical and diagnostic implications of monoclonal B-cell lymphocytosis. *Best Pract Res Clin Haematol* **23**, 61-69 (2010).
  49. Greig, B., Oldaker, T., Warzynski, M. & Wood, B. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: recommendations for training and education to perform clinical flow cytometry. *Cytometry B Clin Cytom* **72 Suppl 1**, S23-33 (2007).
  50. Euroflow. Flow cytometry for fast and sensitive diagnosis and follow-up of haematological malignancies. at <<http://www.euroflow.org/presentation.php>>



51. Pedreira, C.E. et al. Generation of flow cytometry data files with a potentially infinite number of dimensions. *Cytometry A* **73**, 834-846 (2008).
52. Pedreira, C.E. et al. A probabilistic approach for the evaluation of minimal residual disease by multiparameter flow cytometry in leukemic B-cell chronic lymphoproliferative disorders. *Cytometry A* **73A**, 1141-1150 (2008).
53. Zuba-Surma, E.K., Kucia, M., Abdel-Latif, A., Lillard, J.W. & Ratajczak, M.Z. The ImageStream System: a key step to a new era in imaging. *Folia Histochem. Cytobiol* **45**, 279-290 (2007).
54. McGrath, K.E., Bushnell, T.P. & Palis, J. Multispectral imaging of hematopoietic cells: where flow meets morphology. *J. Immunol. Methods* **336**, 91-97 (2008).

For Peer Review