CHAPTER

2

FCM immunophenotyping and DNA analysis: Practical aspects that can affect data analysis and interpretation

In the optimal setting, the FCM lymphoma–leukemia immunophenotyping laboratory is an integral component of the diagnostic hematopathology service. Flow cytometric analysis involves three stages: preanalytical (specimen handling and processing, including antibody staining), analytical (running the sample through the flow cytometer and acquiring data), and postanalytical (data analysis and interpretation). The quality and performance of the preanalytical and analytical steps impact on the resulting fluorescence data and thereby the interpretation. Deficiencies such as suboptimal instrument performance, poor reagent quality (antibodies and/or fluorochromes), or poor specimen quality can all result in inadequate resolution of positive and negative immunofluorescence.

Integrating the FCM and hematopathology laboratories facilitates both the preanalytical steps (because the specimen can be processed simultaneously for other related technologies) and the postanalytical steps (during which the FCM results are correlated with other data prior to establishing a diagnosis). The methodologies used in the authors' laboratories follow closely the recommendations of the 1997 U.S.–Canadian Consensus on the Use of Flow Cytometry Immunophenotyping in Leukemia and Lymphoma. The general aspects of the methodologies for the preanalytical and analytical steps are presented in this chapter. Discussions on quality control are not included, however, as they have been presented at length in previous textbooks and manuals.

2.1 Sample selection

The laboratory has little control over certain factors, such as specimen collection and transportation, which can adversely affect the sample prior to its arrival. Although rigorous quality control applied to the various intralaboratory procedures can ensure the accuracy and reproducibility of the FCM results, poor specimen collection remains a major source of potential unsatisfactory FCM analysis. The time elapsed between specimen acquisition and delivery to the laboratory, and the environmental conditions during transport are critical factors affecting the viability of the cells in the sample. As a rule, specimens cannot be held for more than 48 hours in the fresh state after collection. This time window is much narrower for samples harboring a tumor with a high turnover rate (e.g., Burkitt lymphoma). For these reasons, specimen requirements and acceptance guidelines should be thoroughly communicated to the clinical services as well as to referring institutions. Exposure to extreme temperatures and the presence of blood clots (or gross hemolysis) are conditions that can render a blood or bone marrow specimen unacceptable for analysis.

Fresh specimens for FCM processing and analysis fall into two broad categories: liquid samples (peripheral blood, bone marrow, body fluids) and solid tissue (lymph nodes, tonsils/adenoids, spleen, bone marrow biopsies, and extranodal infiltrates). The size and cellularity of the sample, as well as the viability of the cells therein, are the main factors determining the final cell yield available for FCM study. Lower cell yield limits the number of markers that

can be analyzed. In such instances, knowledge of the clinical setting and prior FCM results are critical in choosing the appropriate markers to be tested.

2.1.1 Liquid specimens

Peripheral blood can be collected in either ethylenediamine tetraacetic acid (EDTA) or heparin. Collection in EDTA is preferred, however, because a hemogram and a blood smear can be obtained from the same sample. The volume of blood required depends on the white blood cell (WBC) count; 10 mL of blood is adequate in most instances. The blood specimen is preferably maintained at room temperature. Referred blood specimens from outside institutions should be accompanied by a hemogram and a fresh blood smear (i.e., free of storage artifacts), either unstained or stained with Wright-Giemsa, for cytologic evaluation. For quality control, however, the FCM laboratory should also make a smear from the FCM blood sample.

Similarly, EDTA is the anticoagulant of choice for bone marrow specimens sent to the FCM–hematopathology laboratory. Bone marrow smears, cytochemistries where appropriate, and FCM studies can all be performed from the same tube. Ideally, the hematopathology staff should be personally involved with the collection of the bone marrow specimen to ensure that the sample obtained is adequate enough to cover all the desired tests (e.g., FCM, cytogenetics, morphology). Two of the authors routinely performed the bone marrow procedure themselves. Using the previously described recommended approach (Nguyen and Diamond, *Diagnostic Hematology: A Pattern Approach*), an ample quantity of marrow aspirate and admixed blood is collected in EDTA tubes. The marrow spicules are allowed to rise to the top, from where they can easily be harvested virtually free of blood contamination and allocated in appropriate amounts for FCM, morphologic smears, and any other necessary studies. This optimal approach eliminates one of the most frequent problems encountered in the laboratory, the marked discrepancy between the cellular bone marrow smears made at the bedside and those made from the aspicular, severely hemodilute marrow sample submitted for FCM analysis. Referred bone marrow aspirate samples received from outside institutions should be accompanied by a fresh bone marrow smear containing an adequate number of spicules. Preferably, a fresh blood smear and hemogram should also be included, so that a complete diagnostic evaluation of the bone marrow can be carried out properly. Approximately 3 to 5 mL of representative marrow aspirate is usually sufficient for a comprehensive FCM analysis, unless the marrow is severely hypocellular. Because bone marrow aspirates have a much higher cell density than peripheral blood specimens, degenerative changes tend to occur more quickly. Refrigeration and the addition of nutrient media containing serum proteins to the aspirate will help to maintain cell viability in bone marrow samples that cannot be processed soon after collection.

Once received in the laboratory, the bone marrow sample is poured out onto a Petri dish to check if spicules are present. A small portion of the spicules is taken to prepare an "in-house" marrow smear for quality control. Two scalpels are applied to mince the remaining spicules to release cellular elements (especially neoplastic lymphoid cells and plasma cells) that tend to adhere to the spicules.

In the case of an aspicular aspirate ("dry tap"), the FCM marrow sample should consist of at least two 2-cm-long core biopsies submitted in sterile tissue culture media, preferably RPMI supplemented with fetal calf serum and a mixture of antibiotics. Cell suspensions can be obtained from the biopsies by the same mechanical dissociation procedure applied to solid tissue.

In body cavity effusions, it is important to collect samples with good cell viability. This can be achieved by draining off the existing effusion, and later obtaining the reaccumulated "fresh" effusion.

For deep-seated lesions (e.g., mediastinal or retroperitoneal), the preferred specimen for FCM analysis is a fine-needle aspiration rather than a core biopsy. Multiple passes (at least 3 to 4), performed by an experienced person, usually provide a higher cell yield than a tissue core biopsy, and minimize sampling error. Body fluids such as CSF, vitreous humor or pericardial fluid carry a scant number of cells and do not require much initial processing. A cell count can be obtained and cytospins prepared directly from the submitted sample.

2.1.2 Solid tissue specimens

Solid tissue specimens (e.g., lymph nodes, spleen, tonsil/adenoids, or extranodal sites) should be submitted as thin slices, less than 2 mm thick, in a generous amount of sterile tissue culture media at 4ºC (i.e., on ice). This helps to reduce the rate of autolysis and degradation of cellular proteins and DNA. For in-house cases, where specimens are delivered immediately to the laboratory, it is only required to keep the tissue moistened with a culture media- (or saline-) soaked gauze. There are no set rules for the required amount of sample because this is dependent on several factors, including the cellularity in the sample, the fragility of the cells, and the susceptibility to apoptosis. More is always better, especially in the case of extranodal specimens where there may be a significant proportion of nonlymphoid tissue or fibrocollagenous stroma. It is important, however, to submit a generous amount of fresh solid tissue to the FCM–hematopathology laboratory, so that an adequate amount of sample may be allocated to various other procedures in addition to the preparation of cell suspensions for FCM analysis. These procedures, potentially necessary for the complete characterization of a particular lymphoid tumor, include the following:

- Snap-freezing for immunohistochemistry or molecular genetics.
- Fixation in a 1 : 1 mixture of RPMI and ethanol for molecular genetics (optional).
- Wright-Giemsa-stained air-dried touch imprints for cytologic evaluation. The cut surface of the tissue slice should be blotted to remove excess fluid prior to making imprints. The imprints would be otherwise unreadable as slow drying produces severe shrinkage artifacts on the cellular elements.
- Fixation for histology, preferably with B-5 or a fixative with a heavy metal component (e.g., barium chloride) for morphologic correlation. For referral cases from an outside pathology laboratory, a small fraction of the sample submitted to the FCM laboratory can be used for histology (if possible). Efforts should be made to obtain H&E sections from the referring institutions, so as to achieve immunophenotypic-morphologic correlation in the FCM hematopathology reports.

To ensure that the sample is representative, the slices sent to the FCM laboratory should be adjacent to those submitted for routine histology (Figure 2.1). A practice to be avoided is that of submitting the tip of the lymph node for FCM while allocating the central portion for routine histology. It is also not advisable to hold the fresh tissue for FCM analysis until after the histologic sections are ready, because the time delay often adversely affects the viability of the sample.

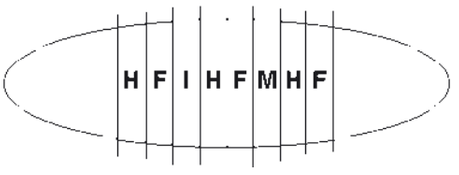

Figure 2.1 Diagram of lymph node slicing and the allocation of the slices to different studies. F, FCM analysis; H, histology; I, immunohistochemistry; M, molecular studies. Each slice is less than 2 mm thick.

Cell suspensions are obtained from the solid tissue by mechanical dissociation whereby the tissue is minced with two scalpels in a Petri dish containing a small volume of culture media, and then passed through a fine-wire-mesh screen. Alternative techniques, such as repeated aspiration of the tissue using an 18-gauge needle or scraping the cut surface of the tissue section with a scalpel blade or glass slide at a 45-degree angle, can also be employed.

2.2 Preparing nucleated cell suspensions

Separating nucleated cells from red blood cells in liquid specimens is achieved by red cell lysis (using ammonium chloride or other solutions). The ghost red cells are removed in the subsequent washing steps. White cells can be stained with antibodies prior to or after red cell lysis. In rare instances, red cells fail to lyse. This may be the result of increased numbers of reticulocytes (e.g., specimens harboring a red cell disorder such as a hemoglobinopathy or thalassemia) or increased lipids in the serum. The unlysed red cells can be electronically removed from analysis, however, by using antiglycophorin antibody and a gating procedure. The red cell lysis procedure is preferred to density gradient methods (e.g., Ficoll-Hypaque) because it allows cells to be maintained close to their native state. Density gradient methods are based primarily on the buoyant density of normal lymphocytes. Because neoplastic cells do not necessarily share the same density as normal lymphocytes, the density gradient methods, despite their ability to remove erythrocytes, mature granulocytes, and dead cells, can result in excessive loss of critical cells. This effect is especially undesirable for samples with a low content of neoplastic cells. In addition, selective population losses in the CD8 subsets can also occur with density gradient techniques.

2.3 Cell yield and viability

Following the red cell lysis step, the cell yield is determined with an automated cell counter. Cytospins are made from the cell suspension to serve as morphologic controls, to ensure that the critical cells have been retained for analysis. When necessary, it is helpful to prepare additional cytospins for cytochemical stains (e.g., myeloperoxidase, nonspecific esterase). The viability of the cell suspension can be assessed by FCM, based on the uptake of DNA-binding dyes such as propidium iodide, 7-amino-actinomycin D (7-AAD) or TOPRO-3 by dead cells. A manual alternative technique using hematocytometer counting is trypan blue exclusion. As a rule, the cell yield and viability tend to be lower in aggressive tumors composed of large cells than in low-grade tumors composed of small cells. The larger neoplastic cells are more fragile and therefore more susceptible to damage and cell loss during the washing and centrifugation steps of specimen processing. Solid tissue specimens often have lower viability than liquid specimens in which most of the cells are already in a disassociated state and exposed equally to the surrounding nutrients. There is no set rule concerning the viability level below which a specimen yields uninterpretable data and therefore becomes unacceptable for FCM analysis. A lower viability can be better tolerated in specimens composed of nearly all neoplastic cells than in samples with a scanty proportion of tumor cells.

2.4 Sample staining

Sample staining should be carried out as soon as possible after the nucleated cell suspension has been prepared. Delaying this step will only reduce viability and induce cell clumping,

especially if the tubes holding the cell suspensions are stored in an upright position. With the exception of cases with low cell yield, a portion of the cell suspension should be kept aside for potential repeats or add-on testing.

2.4.1 Surface antigens

The multicolor direct immunofluorescence-staining technique using commercially available antibodies is employed for the simultaneous detection of multiple cell surface markers. Cell surface antigen staining is performed on viable unfixed cells. All staining is performed at 4ºC to minimize capping and antigen shedding. Appropriate isotype controls are included. The usual number of cells recommended for immunostaining is 10^6 cells for each test (i.e., each tube of antibody reagent cocktail). In situations with low cell yield, it is possible to perform the staining with as few as 1×10^5 to 2×10^5 cells/tube, however. The procedure should be carried out gently so as to minimize any further cell loss.

An efficient and cost-saving strategy is the use of a microtiter plate-based method, which reduces the number of cells required for each test along with the volume of reagents, while maintaining the same proportions as in the conventional tube method. Each well of the microtiter plate requires only one-fifth of the reagents and cells. Batches of antibody reagent panels in microtiter plates can be prepared in advance, stored frozen, and thawed for use as needed. The microtiter plate method is further enhanced by the use of automated cell handlers. Computer-controlled devices to resuspend and introduce cells into the fluidic system of the flow cytometer are currently available, thus achieving a highly efficient and virtually "hands-free" operation.

2.4.2 Intracellular antigens

Although the testing for intracellular antigens is performed up front in some laboratories, the authors' preference is to have these done as "add-on" tests when it is medically necessary (*see* Section 2.8). The staining procedure is more laborious than cell surface antigen staining and calls for cell fixation and permeabilization. Cells are fixed to maintain structural integrity and are permeabilized to allow antibodies to reach the appropriate intracellular targets. The fixative should preserve the epitope of the intracellular antigen in question without causing aggregation of the cell suspension. Intracellular targets include TdT, cytoplasmic light chains, cCD3, cCD22, myeloperoxidase, bcl-2, cyclin-D1, and Zap-70. For increased sensitivity, the detection of intracellular antigens is done in conjunction with cell surface antigens (e.g., CD38 [for cytoplasmic immunoglobulins], CD19, or CD10 [for TdT]). To evaluate surface and intracellular antigens simultaneously, the cell surface antigens are stained first, followed by the fixation and permeabilization step, then staining of the intracellular marker. As with all staining procedures, appropriate background controls are included. In addition, a cell line expressing the targeted intracellular antigen is run in parallel with the patient's sample.

It may be necessary to empirically determine the optimal staining conditions for the various surface antigen(s)–intracellular antigen combinations to ensure the stability of the antigen-antibody-fluorochrome complexes on the cell surface and the preservation of the targeted intracellular antigens. Because the success of intracellular antigen staining depends on the use of small-sized fluorochromes, antibodies targeted against intracellular antigens are most often conjugated to fluorescein isothiocyanate (FITC). Other fluorochromes, such as phycoerythrin (PE), may be used for simultaneous surface antigen labeling. In the authors' laboratory, this staining technique was also applied to the simultaneous analysis of a surface antigen and DNA content using FITC and propidium iodide (PI) labeling, as there is a good separation between the emission signals of these two fluorochromes. The recent advent of DRAQ5 (deep-red

fluorescence bisalkylaminoanthraquinone no. 5) has replaced PI and alleviated the fixation and permeabilization steps for this procedure, however.

2.4.3 DNA content

In the authors' laboratory, DNA ploidy and cell cycle analysis are routinely assessed in acute lymphoblastic leukemia–lymphoma and in tissues involved by LPD/NHL. Prior to the millennium, the analysis was carried out with PI as the DNA dye. In selective instances where the proportion of ALL cells was low (e.g., partial involvement of the peripheral blood or bone marrow), DNA analysis was performed in tandem with TdT or cell surface antigen (e.g., CD19) staining. A similar approach was applied to solid tissues with partial involvement by LPD/NHL where DNA analysis was gated on the critical cells by utilizing the cell size (FSC) parameter or, when necessary, the concomitant staining for a surface label. In recent years, however, DRAQ5 has replaced PI as the DNA dye of choice. As a result, tandem staining with two surface antigens has become the norm (i.e., irrespective of the proportion of neoplastic cells in the FCM sample) in the author's laboratory.

DNA staining is performed on fresh cell suspensions. A large number of cells need to be analyzed to obtain robust measurements of the S-phase, an area that contains relatively fewer cells than the other phases of the cell cycle. The staining procedure is started promptly after the nucleated cell suspension is ready to minimize degradation of DNA. DNA degradation can alter the stoichiometry of dye binding to DNA, thereby affecting interpretation. The authors do not recommend DNA analysis on formalin-fixed, paraffin-embedded tissues. Although this approach makes retrospective studies possible, it often results in poorer quality analysis and an apparently higher S-phase as an artifact. Separation of normal lymphocytes closely admixed with tumor cells cannot be performed reliably on fixed tissue. Furthermore, the archival material precludes simultaneous DNA and cell surface antigen staining.

There exist a variety of DNA-binding fluorochromes with excitation/emission wavelengths ranging from the ultraviolet to the visible regions of the spectrum. Before the availability of DRAQ5, the most common DNA dyes for clinical applications were PI and 7-AAD. The former binds to double-stranded nucleic acids by intercalating between the base pairs. Combining PI staining with RNAse treatment (to eliminate nonspecific staining of folded back single-strand RNA) had produced consistent high-quality results in the authors' laboratory. The permeabilization step utilizes a fixative, either ethanol or paraformaldehyde. Ethanol fixation provides excellent preservation of DNA for long periods of time, but may cause cell shrinkage and loss of cell surface staining. It is best suited for stand-alone DNA staining. Paraformaldehyde fixation is more appropriate for simultaneous antigen detection (using FITC–conjugated antibodies) and DNA analysis, because it can preserve cell light scatter properties and antigen staining. The concentration of paraformaldehyde and the duration of fixation are critical factors to be considered, because a slight excess of this fixative may induce DNA cross-linking, thereby increasing the coefficient of variation (CV) of the DNA histograms. Either PI or 7-AAD can be employed in the simultaneous evaluation of DNA content and surface antigen(s). A limitation of PI is that only one surface antigen can be tested using an FITC–conjugated antibody because the spectral emission of PI overlaps with that of PE, whereas that of 7-AAD does not. A more costly reagent than PI, 7-AAD is also a bulky molecule, leading to the problem of nonstoichiometric DNA binding, which in turn may result in false aneuploidy.

The commercial availability of DRAQ5 in recent years has revolutionized the simultaneous study of DNA with multiple surface antigens. The optimal excitation wavelength for this synthetic fluorochrome is at 647 nm, the emission line of a krypton laser. DRAQ5 is also excitable at the wavelengths emitted by the argon laser (488 nm) and helium-neon laser (633 nm) present

in standard benchtop FCM instruments, however. The emission spectrum of DRAQ5 extends from 670 nm into the far-red visible light, and is therefore distinct from that of FITC, PE and Texas red. DRAQ5 staining can thus be combined with FITC– and PE–conjugated antibodies, and the stained sample can be analyzed without much need of color compensation. DRAQ5 shares some of the desirable features found in PI, namely stoichiometric DNA binding and a low CV, to permit the detection of near-diploid aneuploidy. The most useful feature of DRAQ5 is its rapid penetration into live cells to bind to DNA without the need of fixation or permeabilization, thus preserving the light scatter and antigenic properties of the cells in the sample. This, in turn, facilitates the measurements of cell cycle phases on different cell subpopulations, especially the tumor population, within a heterogeneous sample.

2.5 Data acquisition

The data acquired from the flow cytometer consist of light scatter and fluorescence measurements on single cells suspended in a liquid stream passing single file through a monochromatic light beam produced by a laser. Each cell is an event of a particular light intensity, recorded in an appropriate channel proportional to that intensity.

The light scatter measurements reflect the physical properties of the cells (cell size or internal complexities), whereas the fluorescence data give information on the membrane or intracellular molecules (proteins, DNA) depending on the antibodies and dyes used for labeling the cells. As each dye-labeled cell passes through the light beam, the fluorochrome bound to the cell absorbs light, is excited to a higher energy state, and quickly returns to its relaxed state by emitting a fluorescence signal of a longer wavelength. The fluorescence signals are collected and amplified by photodetectors.

2.5.1 Calibration

The various elements of the flow cytometer, namely light scatter detectors and fluorescence detectors, which include the voltage settings and spectral compensations of the photomultiplier tubes, must be monitored daily to ensure proper data acquisition. Standardized fluorescent beads are used for calibrating the instrument to determine that there have not been significant variations in the instrument settings from day to day. In this procedure, the electronics (i.e., the high-voltage settings of the photomultiplier tubes) are adjusted to place the fluorescence peaks of the beads in the same channels every time, thereby documenting any minor change in the settings. Another set of beads is used to monitor the mean channel and the CV for the FSC, SSC, and each of the fluorescence signals. These parameters are considered to be operating within acceptable limits if the CVs are <3.0 for FSC and SSC and <2.0 for each of the fluorescence signals.

2.5.2 Color compensation

The initial setup of compensation settings is an empirical process, which depends on the fluorochrome combination and the particular instrument (i.e., the optical filters and photomultiplier tubes [PMTs]). Because of the broad emission spectra of fluorochromes, the light collected by an optical filter of a specific wavelength range reaching a PMT detector, consists of not only the signals from the intended fluorochrome but also signals from the other fluorescent dye(s). For example, in the case of an FITC and PE combination, the overlap in their emission spectra is such that a higher percentage of FITC signals are detected by the PE detector than the other way around. To correct this "spillover," a fraction of the FITC fluorescence gets subtracted from the total fluorescence measured by the PE detector. The most appropriate

material for the initial setup and subsequent monitoring of color compensation is a normal control cell preparation, which consists of mononuclear cells from the buffy coat of a unit of donated blood. Each buffy coat can yield approximately 50 to 100 vials of 2×10^7 mononuclear cells that can be stored frozen, then thawed for daily use. The control cells are stained with mutually exclusive markers bearing the fluorochromes of interest, such as FITC–CD4, PE–CD8, and peridinium chlorophyll protein complex (PerCP)–CD20. Compensation adjustments are made while examining the data from each tube on dual fluorescence dot plots. Once compensation has been adjusted for each of the PMTs, the three aliquots are mixed into a single tube and analyzed. Any further modifications to the PMT high voltage or gain setting will require recompensation.

2.5.3 List mode data collection

The goal of data acquisition is to collect measurements (light scatter, fluorescence) from each cell individually. The presence of doublets or aggregates can corrupt the acquired data. The adverse effect of doublet contamination is most serious when the cells of interest are few and the likelihood for confusion with a doublet event is high. The doublets may either originate from the cell suspension or result from an inappropriately high rate of sample throughput. In the latter instance, a doublet is the result of two single particles being so close to each other in the sample core stream that the flow cytometer sees them as a single event. The diameter of the core stream and thereby the alignment of the cells is affected by the sample delivery rate into the flow cell; the higher the sample delivery rate, the wider the core stream and the lower the precision and accuracy of the data collected. Doublets and cell aggregates in the cell suspension can be the result of either delay in analysis or suboptimal tissue dissociation, in which case they are also present on the corresponding cytospins.

When running the sample for DNA analysis, the concentration of cells should be kept high (between 5×10^5 and 2×10^6/mL) and the flow rate low to achieve a low CV. The lower the CV is, the better the measurement of both ploidy and the calculations of the different phases of the cell cycle. In the authors' laboratory, the maximum threshold for the flow rate is 200 cells/second. If the sample contains two different populations with very close DNA contents, the resolution can be improved by lowering the flow rate further. The optimal number of events to be acquired is between 30,000 and 70,000 cells, to achieve adequate statistics for cell cycle determinations. More cells should be acquired when the fraction of neoplastic cells is low. The data are collected ungated (i.e., no data, including debris, is discarded). The extent of debris signals present in the channels below the G_0 peak is a clue to the magnitude of the debris in the channels underlying the S-phase and G_2 peak.

A slightly higher flow rate, 400 to 500 cells/second, is acceptable for immunophenotyping. In the authors' laboratory, 20,000 to 30,000 events are routinely collected ungated from each sample tube after exclusion of the nonviable cells. Low levels (below 1%) of abnormal cells can be detected by an experienced observer when the phenotype is quite specific, such as that displayed by hairy cells. In other instances, where the phenotype is not so pathognomonic, acquiring a higher number of cells will improve the sensitivity of detection. The ungated data collection ensures that no critical cells are missed. After the initial acquisition of ungated data, in selected cases additional data can be collected, gating on the particular minute population of interest (e.g., live-gating to enrich a small population of potentially monoclonal B-cells). All other necessary gating procedures, such as gating on CD45 to identify leukemic blasts, are performed during the subsequent analysis of the originally ungated list mode data. For cases with poor red cell lysis, as evidenced by the presence of abundant RBCs on the cytospin and a sizable cluster of CD45-negative cells with very low FSC, the data collection should be on 20,000 "non-RBC" events.

A different approach to data collection is applied for the assessment of minimal residual disease because the sensitivity of the detection depends on the number of cells analyzed. According to previous studies on MRD (many using PCR-based methods), it appears that the clinically significant level of MRD is at 10^{-4} and above. For assessing MRD by FCM, detailed information on the phenotypic characteristics of the patient's leukemic cells should be available from a previous FCM analysis at the time of diagnosis. Using a set of appropriate markers, the data are acquired with a "live gate." Greater than 10^5 cells need to be collected in order to achieve a sensitivity of detecting one leukemic cell per 10^4 normal cells.

2.5.4 Exclusion of nonviable cells

Flow cytometric identification of nonviable cells is preferably done with PI incorporation, especially for solid tissue specimens, as the viability is often lower than that in liquid specimens (Figure 2.2). Other possible reagents include TOPRO-3 or 7-AAD. An alternative

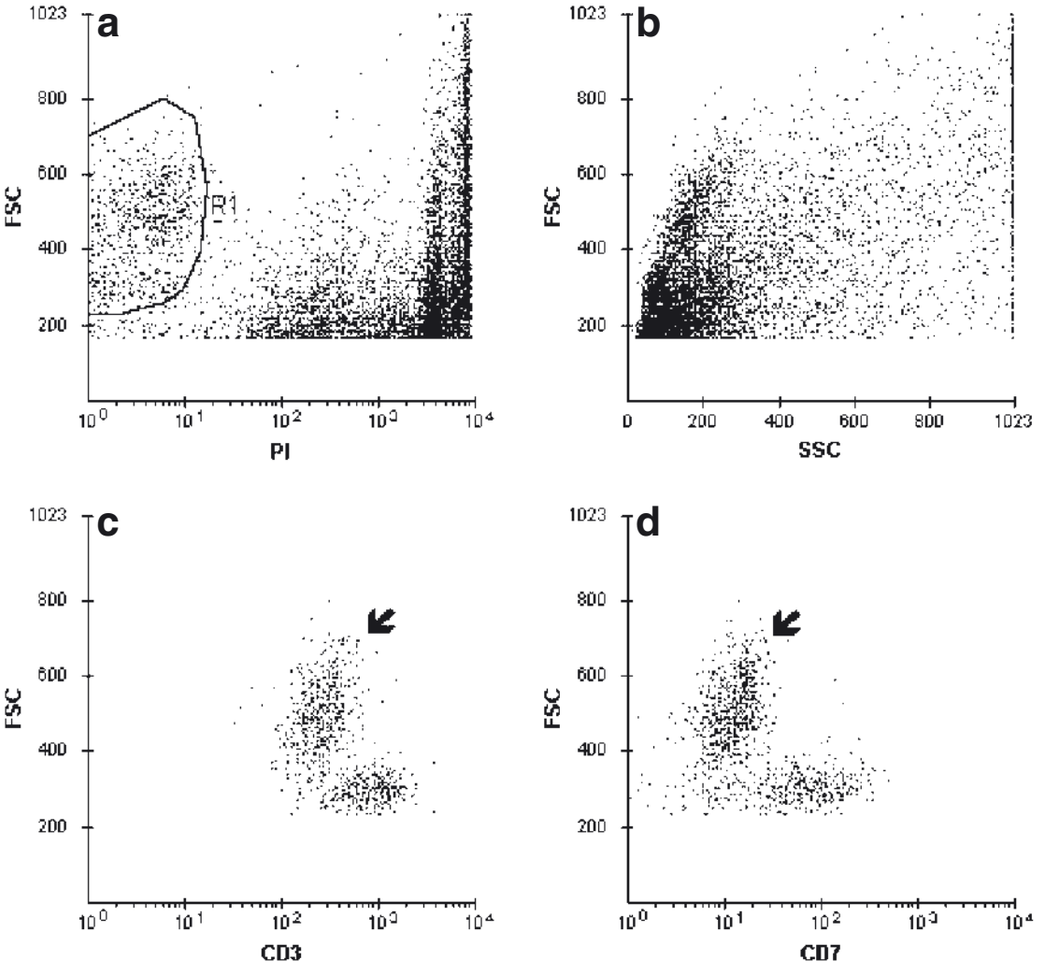

Figure 2.2 Liver with involvement by peripheral T-cell lymphoma. (a) The high uptake of PI by dead and dying cells facilitates the separation of viable (R1 gate) from nonviable cells. The viability in this sample is 15%. (b) The high content of nonviable cells imparts a disarrayed appearance to the ungated FSC/SSC dot plot. (c, d) Gated on R1: The tumor (arrow) demonstrates high FSC, downregulated CD3 and a loss of CD7 expression. Residual T-cells (low FSC, $CD3^+$, $CD7^+$) are present.

method is to gate dead cells out by the forward scatter parameter. Although dead and dying cells appear larger under the microscope, they have a lower refractive index than viable cells and therefore scatter less light in the forward direction. As a result, they can be removed electronically by excluding the lower FSC channels, where dead cells and debris accumulate. This FSC gating approach is less accurate than PI exclusion, however, especially on samples with cell populations of heterogeneous cell size, because the larger dead cells will fall in the same region as the smaller viable cells.

2.6 Antibody panel design

For the analysis of leukemias and lymphomas, the antibodies are assembled into panels. There has been no agreement between laboratories on a uniform and standardized panel. It is not unusual for the panels in use in a particular laboratory to be based on practical (cost concerns and number of samples) rather than medical considerations. The design of antibody panels reflects one of two opposite approaches to the FCM workup of leukemias and lymphomas: comprehensive versus stepwise. In the first approach, the sample is analyzed with a large panel from the start. The number of antibodies in the panel is sufficiently extensive to permit a full characterization of the neoplasm, including any aberrant expression of the cell surface antigens. In the second approach, the FCM study starts with a limited panel. Then, based on the initial results, further analysis with appropriate antibodies is performed on the remaining sample, if necessary. Not infrequently, more than one round of additional testing may be required before reaching a final diagnosis. The stepwise strategy is more economical in terms of reagent cost, but the turnaround time is slower. The high frequency of additional testing can be disruptive to the laboratory workflow, especially in a laboratory with a high volume of FCM specimens. This can end up being expensive in terms of the technologists' efficiency. A more important factor to consider is the decreased cell viability in the sample between the initial and subsequent additional steps, which, in turn, can affect the results adversely. The comprehensive approach, because of its large battery of reagents, is more costly. Using the microtiter plate technique and/or processing a large number of specimens can offset the cost, however. The availability of additional fluorochromes and newer generations of flow cytometers, being more sophisticated and equipped with three (or more) detectors should also help, in the long run, in decreasing the cost by reducing the number of redundant antibodies and shortening the analysis time.

The clinical impression or the morphologic features of the specimens should not dictate the design and selection of an antibody panel. Despite efforts to improve the communication between the clinical and laboratory services, the clinical information on the FCM request forms is often scanty, vague, and potentially misleading. Furthermore, to have an antibody panel for each specific group of hematologic neoplasms (e.g., an ALL panel, AML panel, B-cell LPD panel, and T-cell LPD panel) would be inappropriate and defeat the purpose of FCM immunophenotyping. For instance, the presence of many large mononuclear cells in the blood or bone marrow does not necessarily indicate acute leukemia. Involvement by a large cell lymphoma can give a similar morphologic picture and, consequently, lead to the selection of the incorrect antibody panel (i.e., an acute leukemia panel instead of a lymphoma panel). Conversely, small ALL cells in sheets in the bone marrow can easily be mistaken as involvement by a mature B-cell LPD. Errors in panel selection can be circumvented by the comprehensive approach to FCM analysis, and in the absence of economic constraints, it is preferable to apply such an approach. A certain degree of redundancy of some critical antibodies (e.g., CD34 and CD20) between different tubes is necessary to optimize the sensitivity of FCM analysis and thereby permit the detection of aberrant antigenic expression on neoplastic cells. In the context of a

scanty specimen with a low cell yield, however, the morphologic findings or clinical information can be useful for guiding the selection of a limited battery of reagents.

2.6.1 Antibody selection

Given the current large repertoire of reagents available from various manufacturers, careful antibody selection and panel design is important to achieve the best possible diagnostic information. Several technical factors need to be considered, including the type of antibody (monoclonal vs. polyclonal), antibody isotype (IgG1, IgG2, IgM), antibody clone, dye conjugation, and preparation by the manufacturer (i.e., the stoichiometry between antibody and fluorochrome). Except for the analysis of immunoglobulins (Igs), FCM immunophenotyping relies on monoclonal antibodies. Compared with the polyclonal antibodies used in the early days of immunophenotyping, monoclonal reagents are cleaner, with less background and cross-reactivity. Monoclonal antibodies targeted against the same antigen structure but produced by different manufacturers do not necessarily have similar antibody reactivity, however. A specific example is the difference in reactivity between CD14–Leu M3 (Becton Dickinson), CD14–Mo2 (Beckman-Coulter), and CD14–My4 (Beckman-Coulter). Another factor to consider when constructing a panel is that antibodies (and fluorochrome conjugation) are more likely to be optimized (and thus best suited for a particular brand of flow cytometer) if the reagents and instruments are from the same manufacturer.

One advantage of monoclonal antibodies is consistency in titer and affinity from one lot of antibody of the same clone to the next. Subtle changes in the preparation may alter the reactivity however, which, in turn, may affect the usefulness of the antibody in characterizing a particular disorder. A specific example is anti-CD20. This reagent has a wide dynamic range in fluorescence intensity, a useful feature to discriminate CLL/SLL (chronic lymphocytic leukemia/small lymphocytic lymphoma) from other B-cell neoplasms, as well as distinguishing neoplastic B-cells from a background of benign B-cells. A seemingly minor change in the manufacturer's preparation of the same clone of CD20 antibody may produce a batch of CD20 with decreased brightness and thereby an altered fluorescence dynamic range, with the resulting loss of this important discriminating function.

2.6.1.1 Anti-light chain antibodies

The high specificity of monoclonal antibodies, each recognizing distinctly defined epitopes, can be a disadvantage in the detection of surface Igs. Immunoglobulins have many epitopes and are, therefore, more easily detectable by polyclonal Fab'_2 fragments than by monoclonal reagents. The surface immunoglobulins in some mature B-cell malignancies may not be produced correctly; one or more epitopes may be deleted or altered. Staining with a monoclonal antibody may yield a false-negative result if the reagent happens to be specific for the missing or modified epitope, whereas polyclonal antibodies, especially those with broader specificities, will give a positive result by reacting with the other epitopes of the immunoglobulin.

The reactivities of anti-light chain (kappa, lambda) antibodies can vary widely between manufacturers. To select the optimal brand of kappa and lambda reagents, several candidate pairs are tested at various dilutions with samples from a variety of known disorders, including reactive lymphoid hyperplasia in lymph nodes or tonsils, CLL/SLL, mantle cell lymphoma (MCL), follicular center cell (FCC) lymphoma, and a neoplasm not producing immunoglobulin (e.g., AML). The samples with reactive hyperplasia, in which both light chains are expressed, are used for titration, to determine the optimal dilution for each reagent. For any given brand of kappa and lambda, the optimal dilution is usually the same for both antibodies. In some instances, however, because of preparation and lot-to-lot variation, one of the two light chain

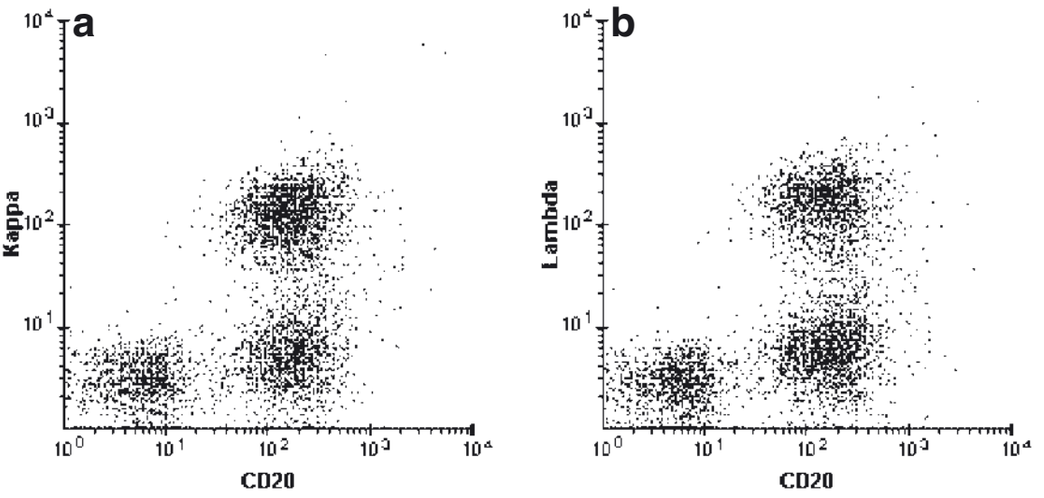

Figure 2.3 Titration of anti-kappa and anti-lambda antibodies using a reactive lymph node. (a, b) CD20-positive B-cells are polyclonal for kappa and lambda. For this particular pair of reagents, kappa had to be one step more dilute than lambda to achieve the desired results.

antibodies may need to be diluted more than the other so as to give identical fluorescence signals (Figure 2.3). Based on this comparative evaluation of different brands of kappa and lambda, a given brand is considered optimal if the fluorescence signals fall in the expected range for any given disorder, namely weak intensity in CLL/SLL and moderate to strong in MCL, along with the least background staining.

With some brands of kappa/lambda reagents, it may not be possible to achieve the appropriate fluorescence intensity. The signals, irrespective of the dilutions, are inappropriately bright for samples with CLL/SLL (Figure 2.4) and may fall in the same fluorescence range observed in FCC and MCL. The intensity of surface light chain expression is one of several criteria critical in the diagnosis and subclassification of mature B-cell malignancies. Therefore, in selecting the optimal polyclonal kappa and lambda antibodies, the appropriate fluorescence signal is a more important consideration than the ability to detect extremely dim light chain expression. Lack of detectable light chains in a mature B-cell population invariably signifies an abnormal or neoplastic B-cell population, with the exception of benign plasma cells and reactive large germinal center cells. For this reason, it is quite unnecessary to conjugate the light chain antibodies to a fluorochrome with a high quantum yield such as PE. Furthermore, because the kappa antibody serves as a control for lambda and vice versa, it is advisable to conjugate these antibodies to the same type of fluorochrome (i.e., FITC), rather then having one light chain conjugated to FITC and the other to PE. In the authors' experience, this strategy is much more sensitive than the reagent kit "kappa–FITC/lambda–PE" provided by the manufacturers. The combination kappa–FITC/lambda–PE may yield ambiguous kappa-lambda results, especially in the context of extreme follicular hyperplasia, or when monoclonal B-cells coexist with a larger population of benign B-cells (*see* Sections 4.1.1 and 4.4.1) (Figures 2.5 and 2.6). Preferably, the antibody panel should contain two sets of kappa and lambda reagents from different manufacturers, each pair combined with a different B-cell marker in the following configuration: kappa (1)–FITC/CD20–PE and lambda (1)–FITC/CD20–PE; kappa (2)–FITC/CD19–PE and lambda (2)–FITC/CD19–PE. This configuration is most helpful in the evaluation of two or more coexisting B-cell subpopulations, benign and neoplastic. Another advantage of this approach is the possibility of assessing the relationship between CD19 and

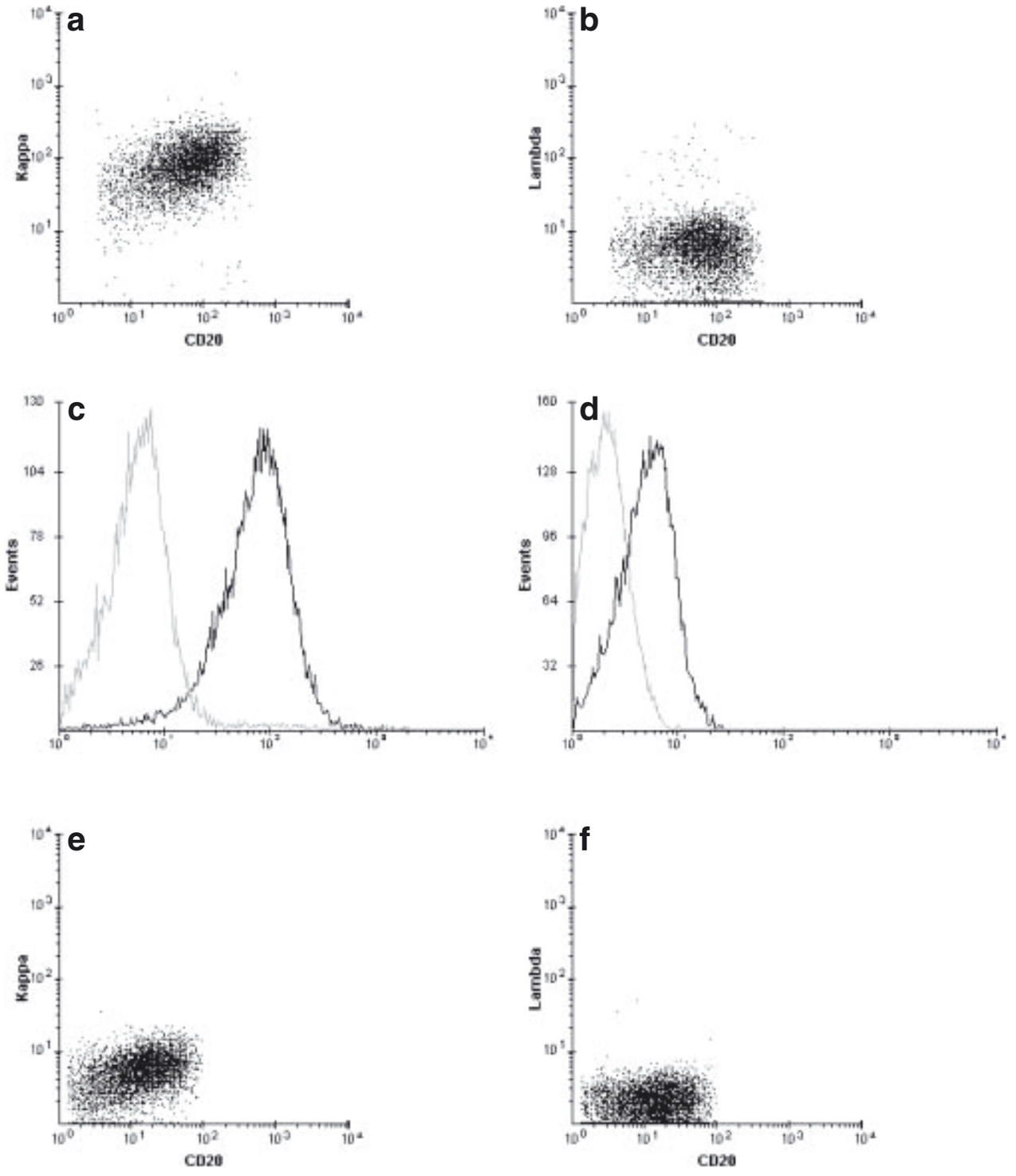

Figure 2.4 Selection of kappa and lambda reagents tested on the same specimen. (a, b) First set of antibodies: The intensity of the positive kappa light chain on CLL cells is inappropriately bright. (c) The peak fluorescence of kappa is 1 decalog brighter than that of lambda. (e, f) The second set of reagents from a different manufacturer yields appropriate results (i.e., dim positive kappa on CLL cells). (d) The same results are seen on the corresponding kappa/lambda overlay histograms.

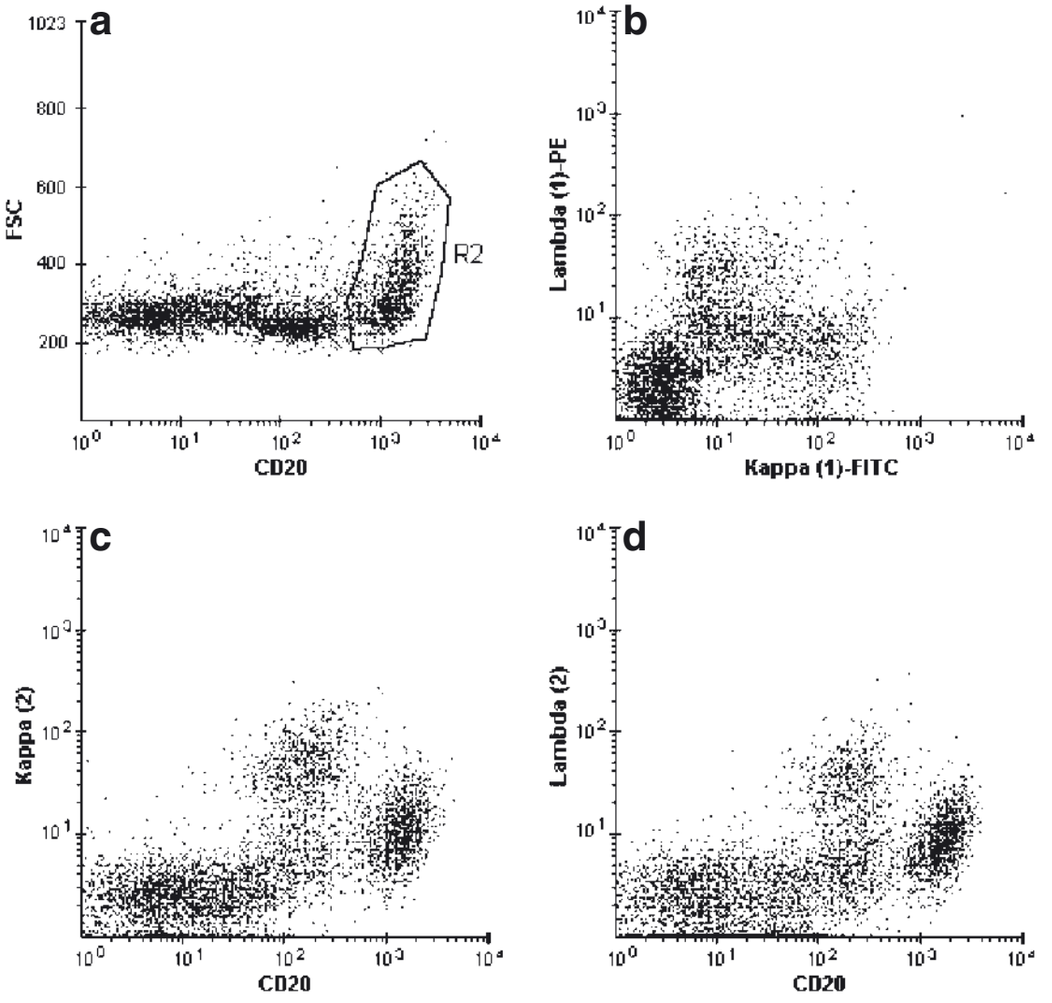

Figure 2.5 Lymph node with FRFH. Benign germinal center cells (R2) display the brightest CD20 intensity (a), but typically lack surface light chains (c, d). The results are clear-cut, using kappa (2) and lambda (2), both labeled with FITC. Staining with the reagent combination kappa (1)–FITC and lambda (1)–PE produces ambiguous results, with an apparent excess of kappa (b).

CD20, a valuable feature in the characterization of low-grade B-cell neoplasms (*see* Section 3.6.2).

2.6.2 Fluorochrome conjugation

For certain antibodies, conjugation to a particular fluorochrome can affect how much information can be derived from the test results. A specific example is the conjugation of FITC to CD10, used in combination with CD20–PE.

The pattern of cell clusters on the CD10–FITC/CD20–PE dot plot is useful for distinguishing the following: (1) normal precursor B-cells (hematogones) from precursor-B ALL cells and (2) follicular lymphoma from florid reactive follicular hyperplasia (FRFH), either of which can present with no detectable light chain. In FRFH, the different cell clusters are seen in close continuity with each other (*see* Section 4.1.1.1). In contrast, when CD10 is conjugated to PE, the dot plot pattern often does not yield the same useful information because the apparent

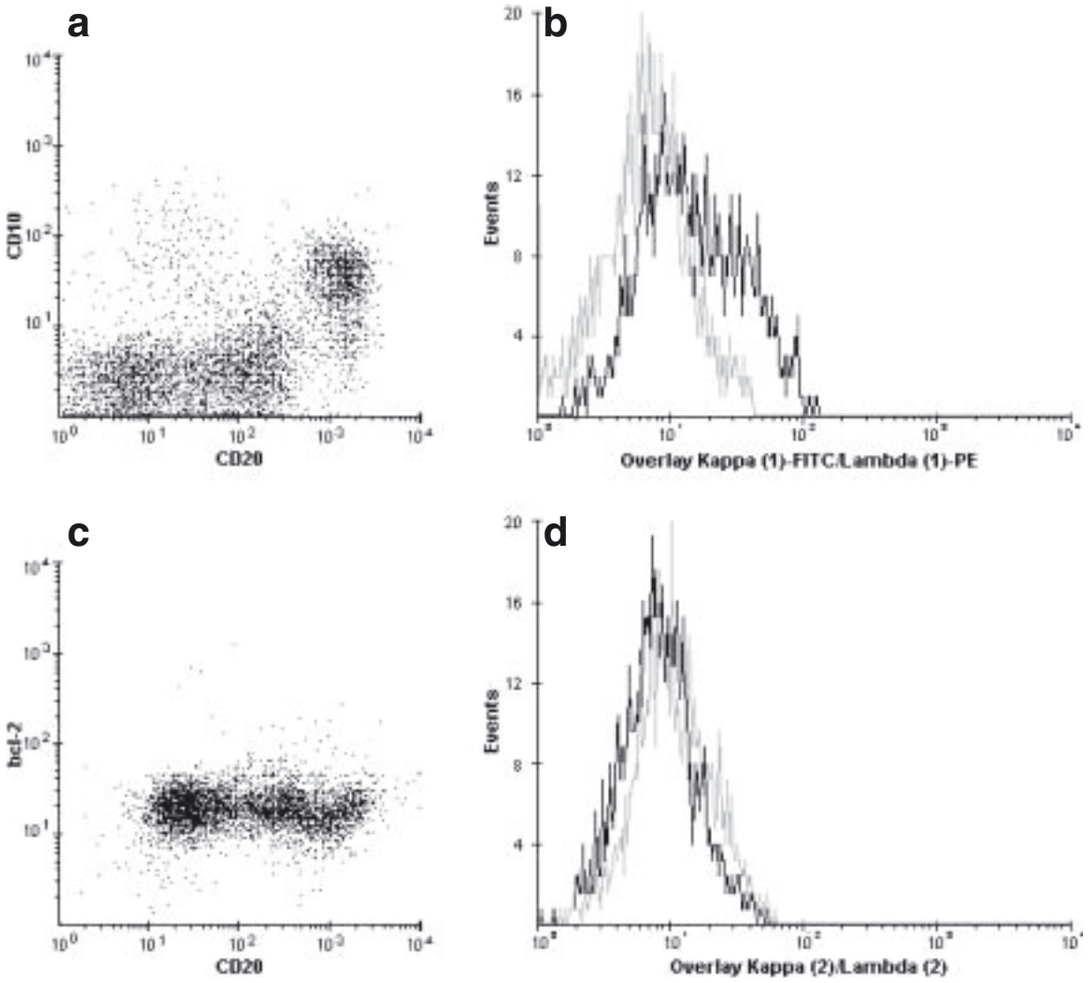

Figure 2.6 Lymph node with FRFH. Benign germinal center cells are CD10 positive (a). bcl-2 is not overexpressed (c). Surface light chains are absent (d). The results are not clear-cut with the reagent combination kappa (1)–FITC and lambda (1)–PE, however (b).

CD10 expression on reactive germinal center cells is much more intense. The resulting double-positive CD10/CD20 cell cluster becomes distinctly separated from the other cell clusters (Figures 2.7, 2.8 and 2.9), an appearance simulating that of FCC lymphoma, which then necessitates additional testing for bcl-2. There exist instances where the conjugation of CD10 to PE is diagnostically useful, however. A specific example is the use of CD10–PE, in combination with kappa–FITC and lambda–FITC for detecting residual/relapsed follicular lymphoma in patients receiving anti-CD20 therapy (rituximab).

The selection of fluorochromes to be conjugated to the monoclonal antibodies (MoAbs) is based on the principle that a fluorochrome with a high quantum yield (e.g., PE, allophycocyanin [APC]) should be used if the antigen sought after is expressed at a low level. Otherwise, the MoAb can be conjugated to a fluorochrome with lower quantum yield, such as FITC. For antigens known to be present at high density (e.g., CD45), the corresponding MoAb can be conjugated to PerCP. For example, CD13 and CD33 should be conjugated to PE so as to maximize the separation of cells expressing these antigens. This principle does not apply to the surface light chains on B-cells, however, because weak expression of monoclonal light chains is a useful diagnostic criterion for the subclassification of B-cell LPD/NHL.

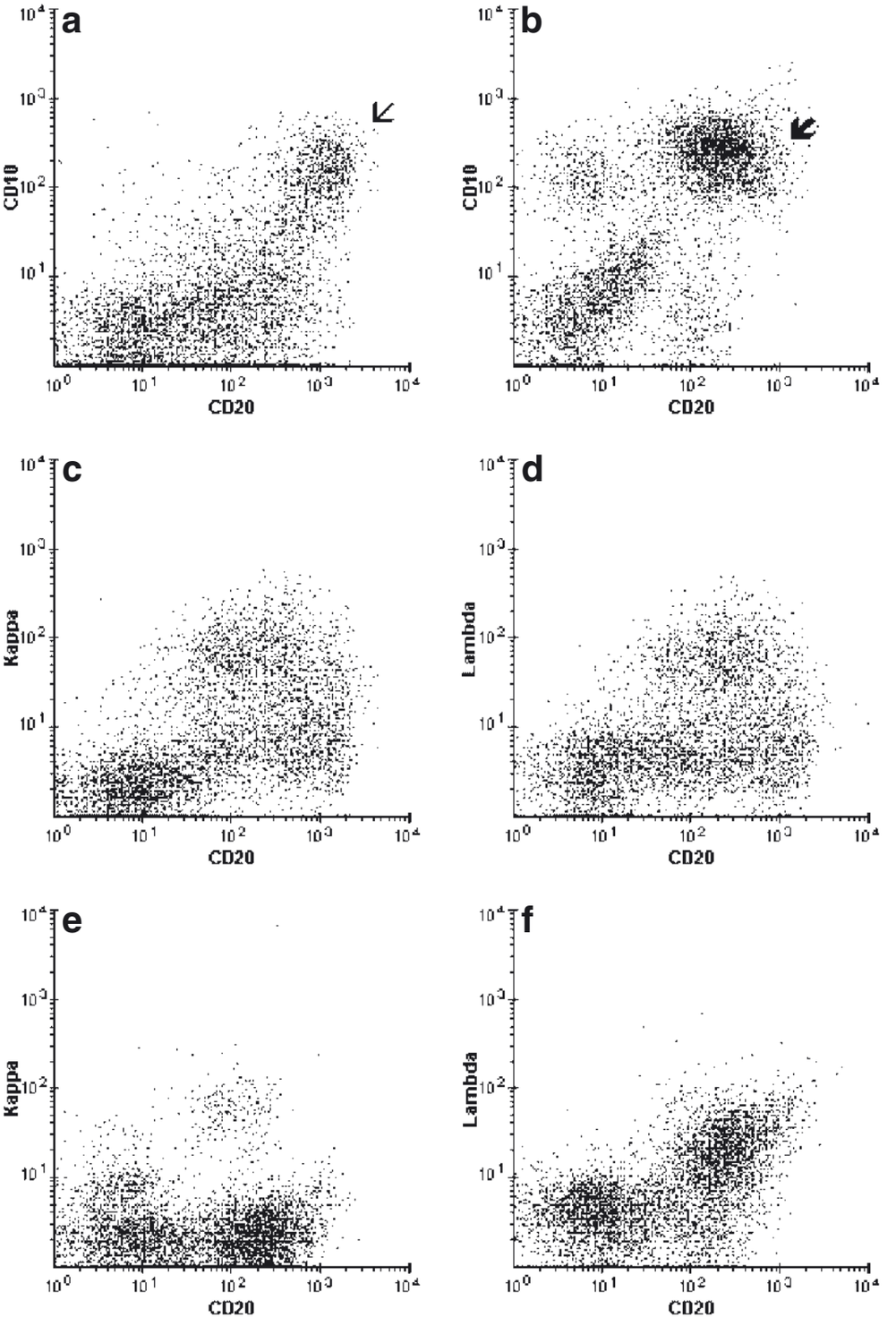

Figure 2.7 Conjugation of CD10 to PE produces an apparent increase in CD10 brightness on the benign germinal center cells (thin arrow) in FRFH (a). The resulting CD10/CD20 staining pattern mimics that on tumor cells (arrow) in an FNA of an FCC lymphoma (b). The latter is monoclonal for lambda (e, f). B-cells in FRFH are polyclonal for kappa and lambda (c, d). The $CD10^+$ $CD20^-$ cluster in the FNA consists of granulocytes (b).

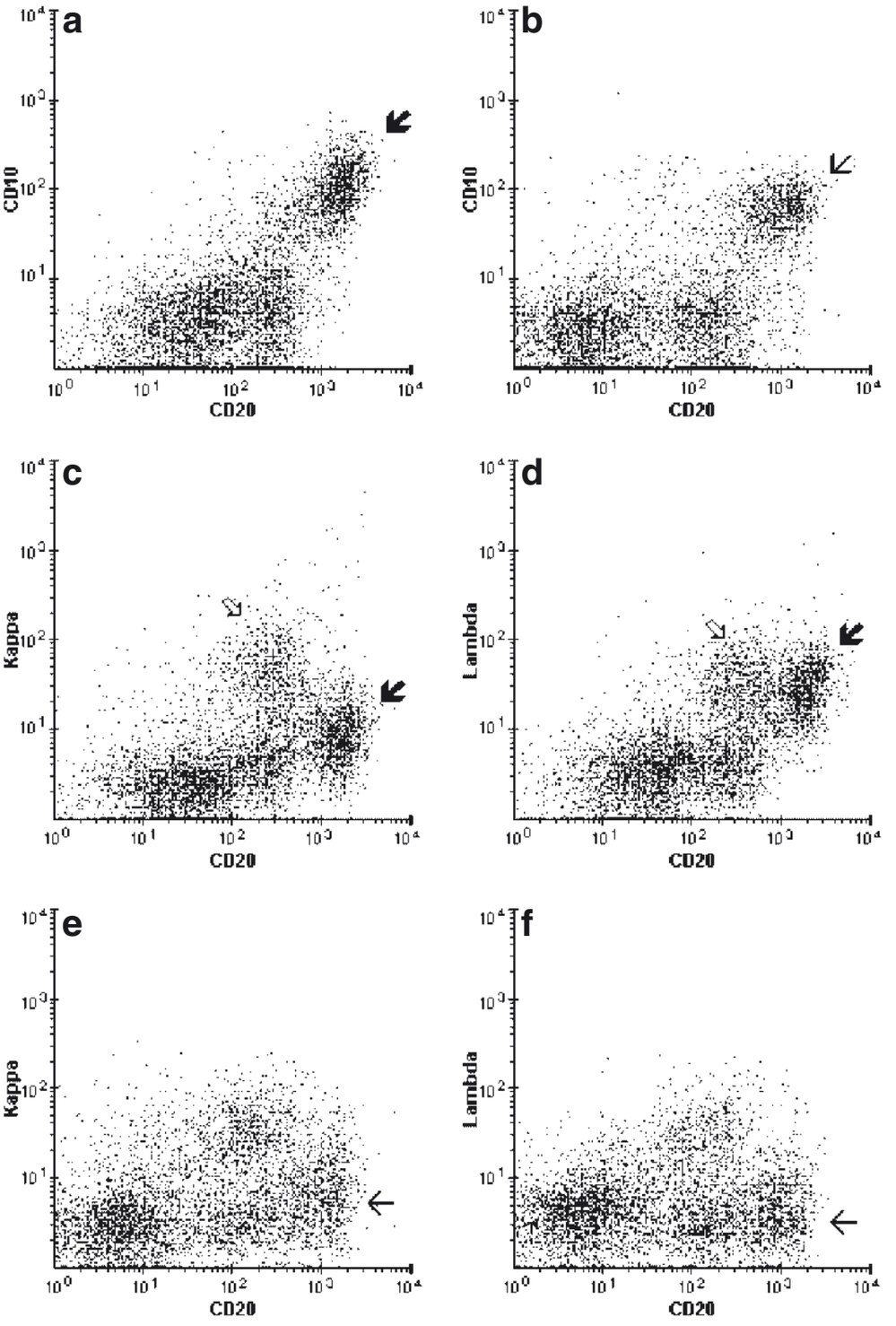

Figure 2.8 Conjugation of CD10 to PE results in a similar CD10/CD20 staining pattern between an FCC lymphoma (arrow) with partial lymph node involvement (a), and benign germinal center cells (thin arrow) in FRFH (b). Residual B-cells are polyclonal (open arrow) and the lymphoma cells are monoclonal for lambda (c, d). Benign germinal center cells show no light chain expression (e, f).

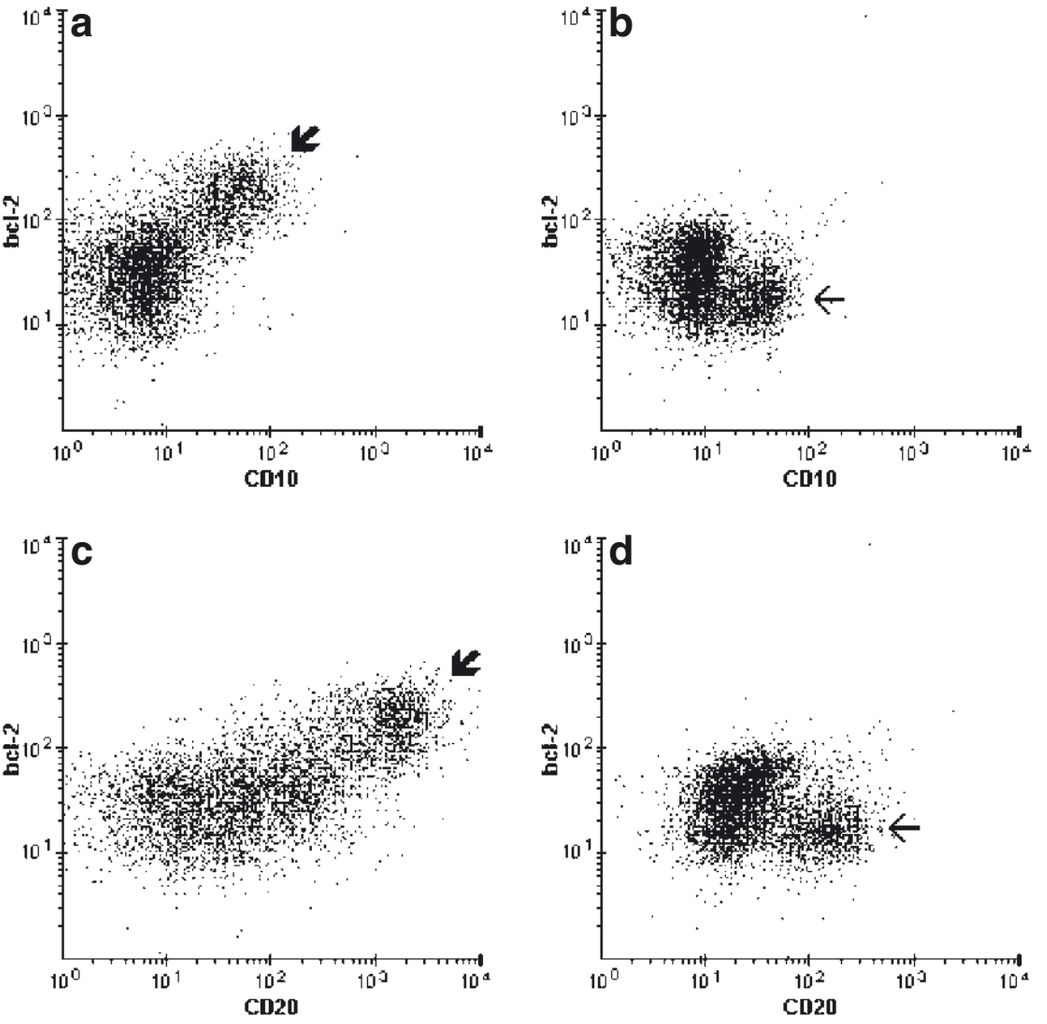

Figure 2.9 FCC lymphoma versus FRFH (continuation of Figure 2.8). Overexpression of bcl-2 in lymphoma cells (arrow) in comparison with residual B- and T-cells (a, c). Germinal center cells (thin arrow) in FRFH are bcl-2 negative (b, d).

The higher the number of fluorochromes for simultaneous analysis, the lower the number of tubes in the antibody panel and the lower the total number of cells required for the entire FCM study; reagents and cells are thus utilized more efficiently. Multiparameter analysis with three or four fluorochromes is currently the norm. Four-color labeling is more desirable if there are no associated technical difficulties. The four-color assay is most suitable for evaluating MRD, where the sample may be scanty and the number of critical cells rare.

When testing solid tissue samples that often contain a significant number of dead cells, it is prudent that one of the dyes be used for excluding nonviable cells. In designing reagent cocktails for multicolor staining, it is important to be aware that one antibody can interfere with the reactivity of another; this can be due to either the isotype of the antibody and/or the type of dye, to which the antibody is conjugated. The electronic process of color compensation is based on the assumption that there is no steric hindrance, enhancement of binding, or dye-to-dye interaction (such as energy transfer or quenching) between the antibodies present in the

cocktail. In general, IgG antibodies are preferable to IgM because there is less likelihood for nonspecific binding and steric hindrance. Similarly, a very large size fluorochrome can cause steric interference.

The authors prefer the single molecule type of fluorochrome, namely FITC, PE, APC, and PerCP to the large tandem conjugates (energy transfer dyes) such as PerCP–Cy5.5, PE–Texas red, or PE–Cy5. Proper energy transfer (i.e., the enhancement of the fluorescence of the acceptor molecule) simultaneous to the quenching of fluorescence of the donor is critical to the function of a tandem conjugate. When there is inefficient energy transfer between the donor and the acceptor molecules, or nonspecific binding to the Fc receptor of monocytes (e.g., PE–Cy5), the use of tandem conjugates may lead to misleading results. The argument for using tandem conjugates is the possibility to perform four-color immunophenotyping on instruments equipped with a single laser (488 nm). Potential problems, namely steric interference and the difficulties in achieving optimal color compensation among the four fluorochromes, especially when two of the four are energy transfer dyes (e.g., PE–Cy5 and PE–Texas red in the cocktail), must be carefully considered in this approach, however. Furthermore, some of the tandem dyes, namely Cy7-containing reagents, require additional precautions during sample preparation and analysis because of their susceptibility to light-induced degradation.

2.7 Comprehensive antibody panels

In the authors' laboratory, the antibody panels have been designed based on a comprehensive approach to FCM analysis. The rationale is to optimize the detection and characterization of the critical cells for determining (1) the lineage of the cells of interest (e.g., myeloid, B-cell, T-cell), (2) their maturity status, (3) the clonality, where appropriate, (4) the specific subtype of hematopoietic malignancy and (5) the status of the normal elements present. The use of large comprehensive panels also facilitates the detection of two or more unrelated neoplastic processes present in the same specimen. Appropriate isotype controls are included in the panels. The evaluation of the FCM data also relies on internal controls, however (e.g., T-cells serve as internal control for B-cells and vice versa) (Figure 2.10).

2.7.1 Disease-oriented antibody panels

Over the years, the panels have evolved to incorporate new monoclonal antibodies of diagnostic significance. In addition, the authors modified the design of the panels from a disease-oriented approach to one based on specimen type. The former strategy includes panels directed toward acute leukemia, lymphoproliferative disorders, or both types of disease. The differences between the acute leukemia and lymphoproliferative panels are that the former includes myeloid markers (e.g., CD13, CD64), whereas the latter contains additional antibodies (e.g., CD25, CD103) necessary for subclassifying lymphoproliferative disorders (LPDs) and lymphomas. Antibodies needed to identify the lymphoid lineages as well as maturity status are present in both panels.

The more comprehensive panel combines all of the antibodies of the acute leukemia and lymphoproliferative panels and is most useful when no relevant clinical information is available, and either the number of critical cells is so low as to escape routine microscopic screening, or the nature of the abnormal cells is undetermined by morphologic criteria. Furthermore, the maturity status of the neoplastic cells (e.g., blasts vs. large lymphoma cells in the blood or bone marrow) may not be apparent on morphologic examination, because critical cytologic features such as nuclear chromatin can be easily altered by a slight degree of suboptimal staining and processing.

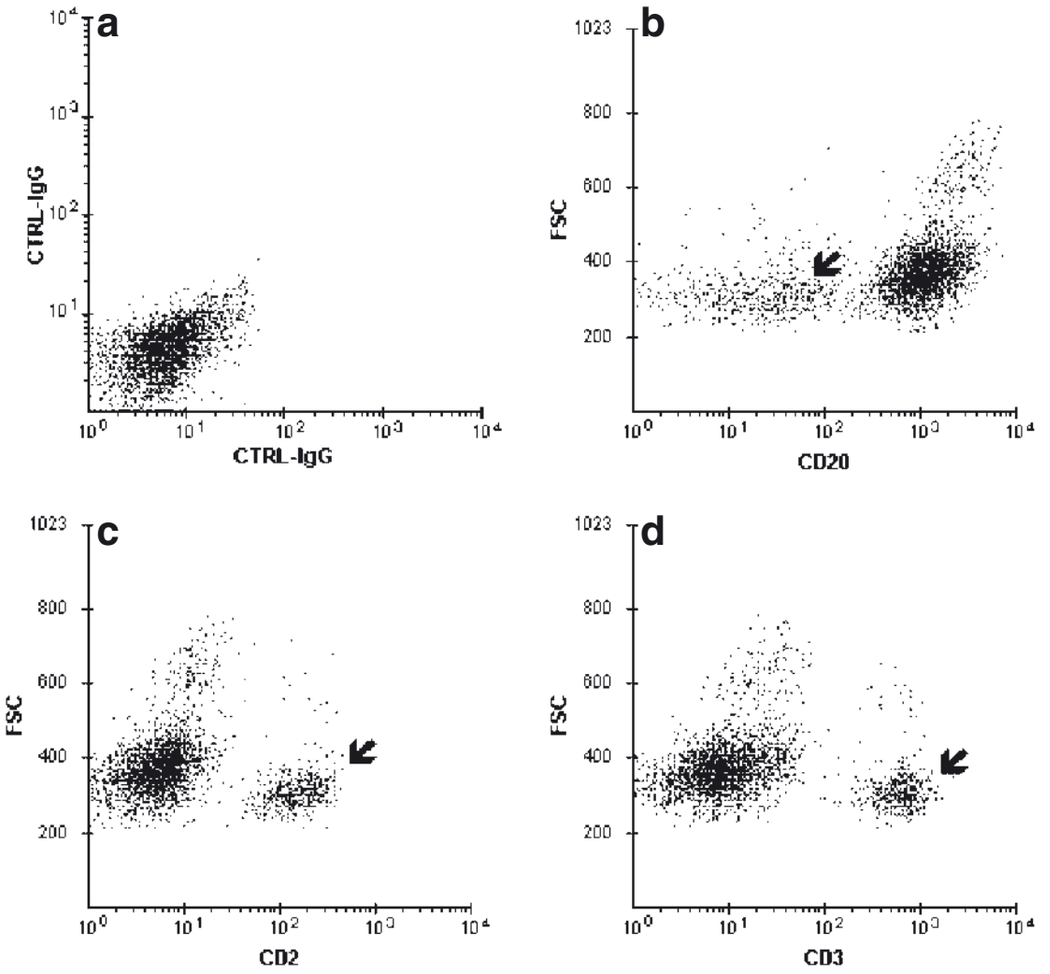

Figure 2.10 Isotype-matched negative controls (a) and internal controls (b–d). The normal residual T-cells (arrow) serve as a negative internal control for CD20, and a positive control for CD2 and CD3. The tumor cells, which comprise the larger cell cluster, are positive for CD20, and negative for CD2 and CD3.

2.7.2 Antibody panels oriented by specimen type

The main drawback to the disease-oriented strategy is that in order to select the proper panel (acute leukemia vs. lymphoproliferative), morphologic screening of the specimen by an adequately experienced professional is required. In the authors' experience from several laboratories (United States and Europe), incorrect panel selection because of morphologic misinterpretation is not an infrequent occurrence. To circumvent these issues, the authors have replaced the above antibody panels with panels oriented by specimen type. Thus, the two new panels, applied since the late 1990s, are a blood/bone marrow/spleen (BBS) panel and a tissue/fluid (TF) panel. This strategy is based on the following rationale: (1) There is an equal predilection for immature hematopoietic malignancies (namely AML and ALL) as well as mature lymphoid neoplasms (B-cell or T-cell LPD/NHL) to involve the blood, bone marrow, and/or spleen, whereas (2) solid tissue and body fluids are much more often involved by lymphoid malignancies (irrespective of the maturity status of the neoplastic cells) than by myeloid neoplasms. In addition, disorders such as plasma cell tumors and hairy cell leukemia (HCL) occur

infrequently in the tissue and fluid compartments. The BBS panel, similar to the combined panel under the disease-oriented strategy, contains a large battery of surface antibodies up front, to permit a full characterization of the neoplasm. The TF panel is smaller, because several of the myeloid-associated markers, as well as CD103 and CD138, are not included. In the unusual event where the initial data reveal involvement of the tissue or fluid by AML, HCL, or plasma cell tumors, then the necessary antibodies are run as add-on tests using portions of the cell suspensions that are routinely set aside for such eventualities.

The design of antibody cocktails for the different "tubes" in the BBS and TF panels, whether in three- or four-color versions (and soon, a six-color version), is aimed at obtaining the maximum diagnostic information from the antibodies. The composition of the cocktails corresponds broadly with the types of cells, normal and abnormal, which may be encountered in any BBS or TF specimen. For instance, the combination CD10 (FITC), CD20 (PE), CD19 (PerCP–Cy5.5), and CD5 (APC) permits the identification of $CD5^+$ B-cells (benign or neoplastic), germinal center cells, or follicular lymphoma cells in lymphoid tissues. In view of the higher frequency of B-cell malignancies (in the Western world) than T-cell disorders, the TF panel is geared more toward B-cells and includes the use of heavy chain antibodies, which gives information on the differentiation stage of any given neoplastic B-cell population. The TF panel also contains one single "myeloid tube" (CD34, CD13/33, CD45, CD14) to cover the eventuality of a myeloid disorder involving the TF compartment (e.g., extramedullary hematopoiesis). In contrast, several myeloid tubes are required in the BBS panel, so as to achieve the full characterization of AMLs and any other abnormalities in granulocytic maturation.

Redundancy in antibodies between "tubes" is necessary for several reasons, including the fact that at least one of the markers serves as an anchor-marker across several tubes. For instance, CD45 is the main anchor marker for most, if not all tubes in the BBS panel. CD20 and CD3 are the secondary anchor markers for the evaluation of mature B- and T-cells in the BBS compartment, respectively. Furthermore, some of the antigens are expressed on more than one cell lineage. For example, CD56 not only helps to identify NK cells and NK-like T-cells, it is also an important marker for the detection of abnormal plasma cells. CD56 is therefore included in one of the T-cell "tubes" (CD7, CD56, CD45, CD3), and in the plasma cell "tube" (CD38, CD56, CD45, CD19) of the BBS panel. In contrast with the BBS panel, the TF panel is not built around a single anchor-marker. Rather the B-cell "tubes" are anchored using CD20 and/or CD19, and the T-cell "tubes" anchored with CD3.

The design of some of the cocktails also takes into account the fact that certain antibody–fluorochrome combinations, namely those with a wide dynamic range, are more informative than others. A specific example is the previously mentioned CD10–FITC/CD20–PE (or –APC) configuration, which has proved to be more useful than its counterpart CD10–PE/CD20–FITC (or –APC) in discriminating germinal center cells from follicular lymphoma cells in the TF compartment. Another important combination is CD11c–FITC/CD20–PE. Because of the wide dynamic range of CD11c, the pattern and the position of the critical cells on the dot plot permits one to distinguish HCL from other $CD11c^+$ B-cell LPDs (*see* Section 3.6.3.2). This combination is especially helpful because a small number of B-cell LPDs may exhibit CD103 reactivity identical to HCL. In general, markers with a wide dynamic range are more useful, as cell populations positive for the same marker can be easily distinguished by their different fluorescence reactivities with that marker. Compared with other B-cell antibodies (e.g., CD19, CD22, FMC-7), CD20 has an optimal wide dynamic range. Therefore, most of the antibodies needed for the characterization of B-cell LPD/NHL are anchored to CD20 instead of CD19. The panels do not include FMC-7 or CD22 because, in the authors' experience, these provide no additional diagnostic information to CD19 and CD20. These antibodies may be helpful for the evaluation of B-cells, neoplastic and benign, after anti-CD20 therapy, however.

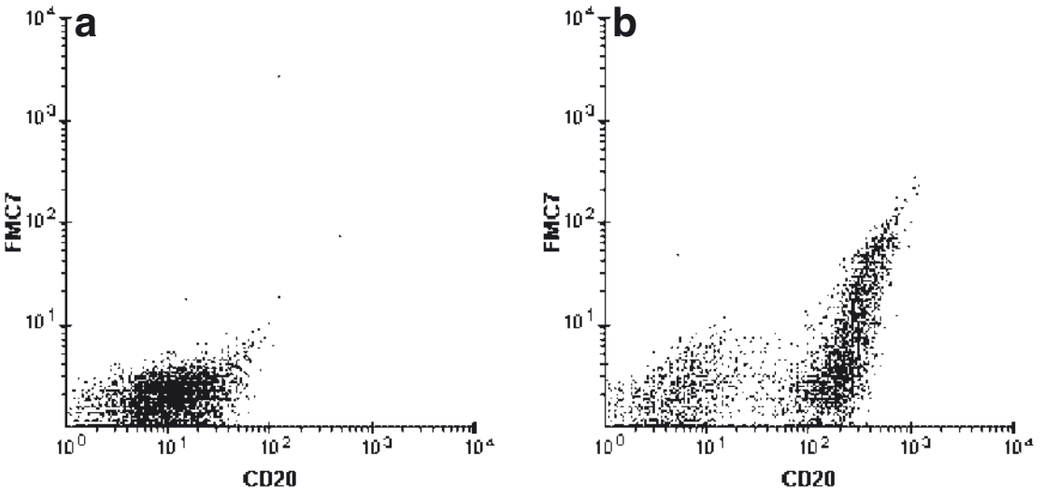

Figure 2.11 Relationship of FMC-7 to CD20. (a) Downregulated CD20 and absent FMC-7 in CLL/SLL; (b) FMC-7 and CD20 coexpression in Burkitt lymphoma.

The main utility of FMC-7 is its absence; lack of FMC-7 expression is a typical finding in CLL. However, this feature is rather redundant for the diagnosis of CLL in light of the characteristic CD20 fluorescence pattern and the relationship of CD20 to CD19 (*see* Section 3.6.2) in this disorder. On a dual fluorescence display of FMC-7 and CD20, a clear linear relationship can be demonstrated (Figure 2.11).

2.8 Tailored panels and add-on testing

In addition to these large routine panels, smaller panels can be tailored to analyze follow-up specimens in patients with a recent diagnosis of hematopoietic malignancy if the original graphical FCM data from the diagnostic sample is available for review. The smaller panel is especially applicable if the follow-up specimen (e.g., CSF, fine-needle aspiration [FNA]) has a low cell yield. For instance, a so-called clonal excess detection panel, which includes CD19, CD20, kappa, and lambda, and a few other markers (CD10, CD5, CD23, CD103), can be applied to follow patients with B-cell LPD/NHL using two or three B-cell tubes. The antibody composition of these tubes can be either identical to their counterparts in the TF panel, or tailored according to the known phenotype of the tumor.

Because of its scantiness, CSF is handled differently from other specimens. Most CSF samples are submitted as follow-up specimens, to rule out involvement by acute leukemia (primarily ALL) or, less commonly, high-grade LPD/NHL. The cytospins may be reviewed first to determine if FCM is applicable. In general, when suspicious cells are present, the selection of the key antibodies to analyze the CSF is based on the FCM results from an earlier diagnostic specimen (e.g., lymph node, bone marrow). Often, the neoplastic cells can be detected by a four-antibody cocktail in a single tube.

Antibodies to detect intracellular antigens (TdT, myeloperoxidase [MPO], cytoplasmic light chains, cCD3, cCD22, bcl-2) are not included in the authors' standard panels. The analysis is performed as an add-on test because antibody staining for intracellular antigens is more time-consuming than that for surface antigens. Because T-cell LPD/NHLs occur infrequently in the

Western world, testing for the surface antigens TCR α/β and TCR γ/δ, NK receptors (CD94, CD161, and KIR antigens) and the T-cell Vβ repertoire are also performed only on a case-by-case basis. Analysis of the Vβ repertoire employs a commercial eight-tube kit, identifying 24 different TCR-Vβ specificities (i.e., about 70% of the normal human Vβ repertoire). The mixture of Vβ antibodies in each tube is designed in such a way that each would react with 10% to 15% of T-cells. Each tube of the kit contains three different Vβ antibodies conjugated to FITC and/or PE, which then allows for two other T-cell markers to be added to the same tube. Note that TCR-αβ cannot be added into any of the tubes of the kit, however. The combined staining for TCR-Vβ/TCR-αβ is not technically feasible, possibly because of steric hindrance. Therefore, to determine the usage of the Vβ repertoire within the TCR-$\alpha\beta^+$ T-cell population, the analysis should include testing for TCR-αβ and TCR-γδ (e.g., using the combinations, TCR-αβ/CD8/CD3/CD4 and TCR-γδ/CD8/CD3/CD4). The selection of which T-cell markers to use depends on the abnormalities detected on the initial FCM run. For example, CD3 and CD4 are employed if the initial data demonstrate a $CD4^+$ T-cell population with either downregulated or upregulated CD3 expression.

To maintain the optimal efficiency in a busy FCM–hematopathology laboratory, the following guidelines can be applied to "automate" the decision of when and which intracellular antigen staining to perform:

TdT: Reactivity with either TdT or CD34 indicates that the neoplasm is composed of immature cells. Therefore, testing for TdT may be omitted for maturity assessment if the leukemia is already $CD34^+$. TdT testing is most appropriate when the results from the standard panels indicate a lymphoid neoplasm with no CD34, no surface light chain expression, and no evidence of plasma cell differentiation. In that case, TdT is necessary to establish the maturity status of the tumor cells, which affects the diagnosis and therapy. A useful approach to assess TdT in ALL is to combine the TdT assay with DNA analysis. Aneuploidy is not only helpful as a prognostic marker but the TdT/DNA combination will also serve as a useful fingerprint for the detection of residual/relapsed disease in the patient's follow-up specimens. Another approach to monitoring MRD of T-ALL or precursor B-ALL is the combination of TdT with T-cell (e.g., CD7, CD3) or B-cell (e.g., CD19, CD10) markers, respectively. The combination TdT/CD19 offers good discrimination between benign B-cell progenitors and residual/relapsed precursor B-ALL (*see* Section 3.5.2).

Whereas the immature neoplastic cells in ALL can be confused morphologically with mature neoplastic lymphoid cells in LPD/NHL, blasts in AML are morphologically distinctive from the maturing myeloid precursors. Therefore, testing for TdT in AML is not necessary irrespective of whether CD34 is expressed or not. The expression of TdT in AML is noncontributory for diagnostic and prognostic purposes. Furthermore, because of the high frequency of antigenic shift in AML, it is unlikely that TdT can be useful as a fingerprint at relapse.

MPO antibody: The demonstration of MPO activity or CD13 and CD33 expression constitutes firm evidence of myeloid differentiation. MPO activity can be detected cytochemically (MPO cyto) or immunologically (MPO Ab). Therefore, MPO Ab testing may not always be necessary if the leukemia is either positive for MPO cyto or expresses both CD13 and CD33. Acute leukemias expressing only one of these two antigens (either CD13 or CD33) but with no lymphoid markers are most likely AML, in which case MPO Ab testing may help to confirm the diagnosis.

MPO Ab testing is most informative when the blast population does not demonstrate a clear lineage (e.g., only one lymphoid and one myeloid marker are expressed). At the same time, staining for the appropriate cytoplasmic lymphoid marker, either cCD22 or cCD3, should also be performed. The frequency of cases where additional testing for MPO Ab and cCD3 or cCD22 is needed is relatively low.

Cytoplasmic CD3, CD22, or mu chain: With the use of multiple antibodies in the panel and the multicolor approach, the need to stain for cCD3 and cCD22 rarely arises in our laboratory. In most cases of T-ALL, the presence of CD2, CD5, and CD7 is sufficient to infer the T-cell lineage.

Previously, cytoplasmic IgM (cmu) was used for subclassifying precursor B-ALL. It is no longer necessary to perform this staining because the presence or absence of cmu has been shown to be of no relevance to prognosis and therapy. Testing for cCD3 may be helpful when the neoplastic cells express fewer than three pan T-cell-associated markers and lack surface CD3 and other lineage markers. Occasional high-grade T-cell lymphomas, in which only CD2 and CD5 or CD2 and CD7 are present, fall into this category.

bcl-2: Testing for bcl-2 in combination with CD20 is appropriate when the results from the TF panel indicate a population of slightly larger cells, $CD10^+$ and $CD20^+$ (intense), and with poor or no surface light chain expression. In these cases, the differential diagnosis is FCC lymphoma versus FRFH. In addition to the pattern of CD10–FITC/CD20–PE coexpression (*see* Section 4.1.1.1), bcl-2 testing helps to resolve the differential diagnosis. This testing is particularly useful in cases of fine needle aspirates or when tissue samples are small and bcl-2 staining by immunohistochemistry may not be easily interpretable.

Cytoplasmic light chains: This assay is performed to assess plasma cell clonality and is most often done in combination with surface CD38 or CD138 staining. Therefore, testing for cytoplasmic kappa (cKappa) and cytoplasmic lambda (cLambda) are performed when the results from the standard panel reveal a relative increase in the number of plasma cells (a distinct population with bright CD38 and negative CD45) but without phenotypic aberrancies. The assay can be omitted if the plasma cells are overtly abnormal, expressing CD56 or CD117. Staining for cytoplasmic light chains can also be paired with CD20 or CD19 to detect lymphoid malignancies with plasmacytic differentiation.

2.8.1 Minimal residual disease

Detection of MRD has been performed primarily in acute leukemias for guiding therapy as well as for prognostic purposes. More recently, it has also been applied to patients with low-grade B-cell malignancies undergoing high-dose chemotherapy, stem cell transplant and immunotherapy, in which case the "clonal excess" tubes are employed for MRD detection. In acute leukemia, MRD assay by FCM analysis takes advantage of the immunophenotypic abnormalities frequently exhibited by leukemic blasts. The abnormalities may be overt, such as the expression of a marker from a different cell lineage, or subtle, in the form of down-regulated or upregulated expression of a number of antigens when compared with normal counterparts. For instance, leukemic cells of most precursor-B ALL can be distinguished from bone marrow B-cell progenitors based on the differences in the expression of several antigens, including TdT, CD45, CD19, CD20, CD10, CD38, CD34 and CD58 (*see* Section 3.5.2). Based on the brighter expression of CD10 and CD58 in the leukemic blasts and using a four-antibody cocktail (e.g., CD10, CD58, CD45, CD19), it is possible to achieve a high resolution of detecting residual/relapsed disease to the level of one blast in 10,000 cells (10^{-4}).

In patients with T-ALL, knowledge of the antigenic profile of the leukemic cells would help to construct a tailored cocktail (e.g., TdT, CD8, CD45, CD4) for MRD detection purposes. The combination of a pan-T-cell antigen with either TdT or CD34 (e.g., TdT/CD3) has also proved to be useful, as combined expression of TdT/CD3 or CD34/CD3 is virtually never encountered in normal bone marrow cells. The presence of other abnormalities such as aneuploidy, the expression of CD56, or an aberrant myeloid antigen, further facilitates the detection of MRD in ALL of either lineage.

The evaluation of MRD in AML by FCM relies on the phenotypic aberrancies (*see* Section 3.5.1.1) present on the patient's leukemic blasts at the time of diagnosis. Based on these abnormalities, combinations of appropriate markers can be selected. Because of the relatively frequent phenotypic changes associated with long-term clonal evolution in AML, the use of such combinations is more suitable for detecting residual disease at the end of induction or consolidation therapy rather than later relapses.

2.9 FCM immunophenotyping data representation

Irrespective of the number of colors used in FCM testing, the standard format for displaying FCM data still consists of two-dimensional (2D) graphics with x- and y-axes. Based on the current commercially available software for FCM data analysis, the most common approach for displaying immunophenotyping data derived from an antibody panel is the automatic tube-by-tube approach. The parameters available from one tube are displayed, followed by those from the next tube, and so on in a sequential manner. The parameters are shown in various permutations for the x- and y-axes, that is, FL1 versus FL2, FL1 versus FL3, FL2 versus FL3, light scatter (FSC or SSC) versus antibody fluorescence, and single-parameter fluorescence histograms. This approach to data display is rather inefficient because not every permutation is informative and there are a high number of graphics being generated.

With some effort and careful planning, however, the FCM software currently available from most manufacturers can be used to create analysis panels based on a medical rationale, which provides a more logical approach to analyzing immunophenotyping data than the tube-by-tube approach. It may be judicious to install the analysis panels on workstation(s) connected via a network, so that the FCM data from any given case can be analyzed at any workstation rather than solely at the computer associated with a particular flow cytometer.

2.9.1 Analysis panels

In the authors' laboratory, one analysis panel has been created for each of the standard antibody panels (BBS, TF). The organization of the FCM displays in the BBS analysis panel follows the rationale that in the BBS compartment, acute leukemias (lymphoid and myeloid) and B-cell NHL/LPD occur more frequently than T-cell disorders. The TF analysis panel reflects a similar reasoning (i.e., that mature B-cell malignancies are more frequent than their T-cell counterparts). Because acute leukemias are considered life threatening, the corresponding displays are among the first to appear on the analysis panels, so as to facilitate the technologist in flagging the case and starting any necessary "add-on" antibody testing based on the above-mentioned "automated" decision-making guidelines. For instance, the BBS analysis panel begins with the SSC/CD45 dot plot, followed by the displays (gated on mononuclear cells) of CD117/CD34, CD13/CD34, CD33/CD34, CD10/CD19, CD10/CD20, CD10/CD58, CD38/CD45 and CD56/CD38. This grouping of dot plots helps one to screen quickly for involvement by myeloid malignancies, neoplastic precursor B-cells or plasma cell dyscrasia.

The preferred format for data representation adopted by the authors is the dot plot whereby each dot corresponds with one event. The printout of the dot plots, especially if done in one color (e.g., in black and white), is best done at the 25% to 50% level of the actual cells acquired to facilitate the visual resolution of closely placed cell clusters.

The analysis panels should be designed to include, in addition to the familiar dual fluorescence dot plots, displays that correlate the cell size (FSC) and antibody fluorescence data. The information derived from these two types of dot plots complement each other. The dot plots for FSC versus antibody fluorescence can be used for displaying ungated cell populations (i.e., in the case of the BBS analysis panel, granulocytes can be included). The dual fluorescence dot plots correlating antibody expressions can be focused on mononuclear cells, in order to identify and characterize the critical cells. Where appropriate, a gate can be drawn around the neoplastic cells (Figure 2.12) so as to estimate their relative proportion. In addition, the BBS analysis panel also includes dot plots gated solely on the granulocytic clusters to evaluate the expression of myeloid antigens (namely CD13, CD16, and CD11b) on the maturing myeloid precursors. The observed pattern of antigenic coexpression is often altered in myeloid

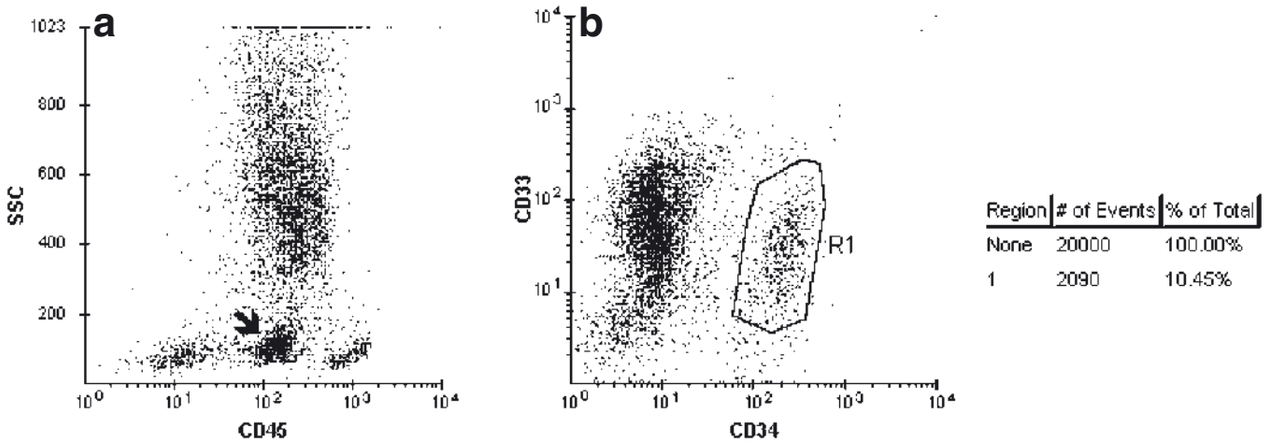

Figure 2.12 Bone marrow with residual AML. (a) The neoplastic cells form a distinct cluster in the blast region (arrow) coexpressing CD33 and CD34. (b) The blast content can be derived from a gate (R1) drawn around the $CD33^+$ $CD34^+$ cluster.

processes, such as some myelodysplastic syndromes and myeloproliferative disorders (*see* Sections 4.2.2.2 and 4.5.2).

2.9.2 Color display

The immunophenotyping data can be displayed in color (e.g., each cell cluster in an SSC/CD45 display can be assigned a color), which may facilitate the identification of cell populations in other 2D projections of the data. Once a given population is depicted with a particular color on the anchor SSC/CD45 dot plot, it will appear in the same color in other dot plots. It is then possible to follow that population from one tube to the next throughout the panel. To use color displays effectively requires that the antibody panel be built around a single anchor-marker such as CD45–PerCP. In other words, the antibody panel cannot have two primary anchor-markers, such as CD45–PerCP in some "tubes" and CD20–PerCP in other "tubes."

The TF antibody panel is not based on any single primary anchor-marker. The graphics of the TF analysis panel are therefore displayed in one color. Where applicable, colors can be assigned to different populations using FSC as the anchor "marker." In the correlated dual fluorescence data displays (e.g., CD71/CD20) (Figure 2.13), the color of the population of interest provides information about its cell size (i.e., small cells vs. large cells). In such instances, the use of color provides a third parameter (FSC) to the 2D fluorescence data displays. This does not preclude displaying the fluorescence data in correlation with the cell size, however. In most laboratories, the emphasis has been on dual fluorescence 2D graphics (e.g., FL1 vs. FL2) and little attention has been given to the correlated FSC and fluorescence data displays. The valuable information (sometimes subtle), which can be derived from the pattern and the relationship of the clusters present on the correlated FSC and antibody fluorescence dot plots, has therefore been overlooked (Figure 2.14).

The main utility of displaying the FCM graphics in color is to reduce the number of FCM graphics to be printed for diagnostic review. The use of color also helps to identify different cell populations, especially those sharing similar reactivity for a given marker. A typical example is the expression of activation markers such as HLA-DR or CD38 by cells of various lineages. For instance, on the dot plot FSC/HLA-DR or HLA-DR/CD34 from a bone marrow

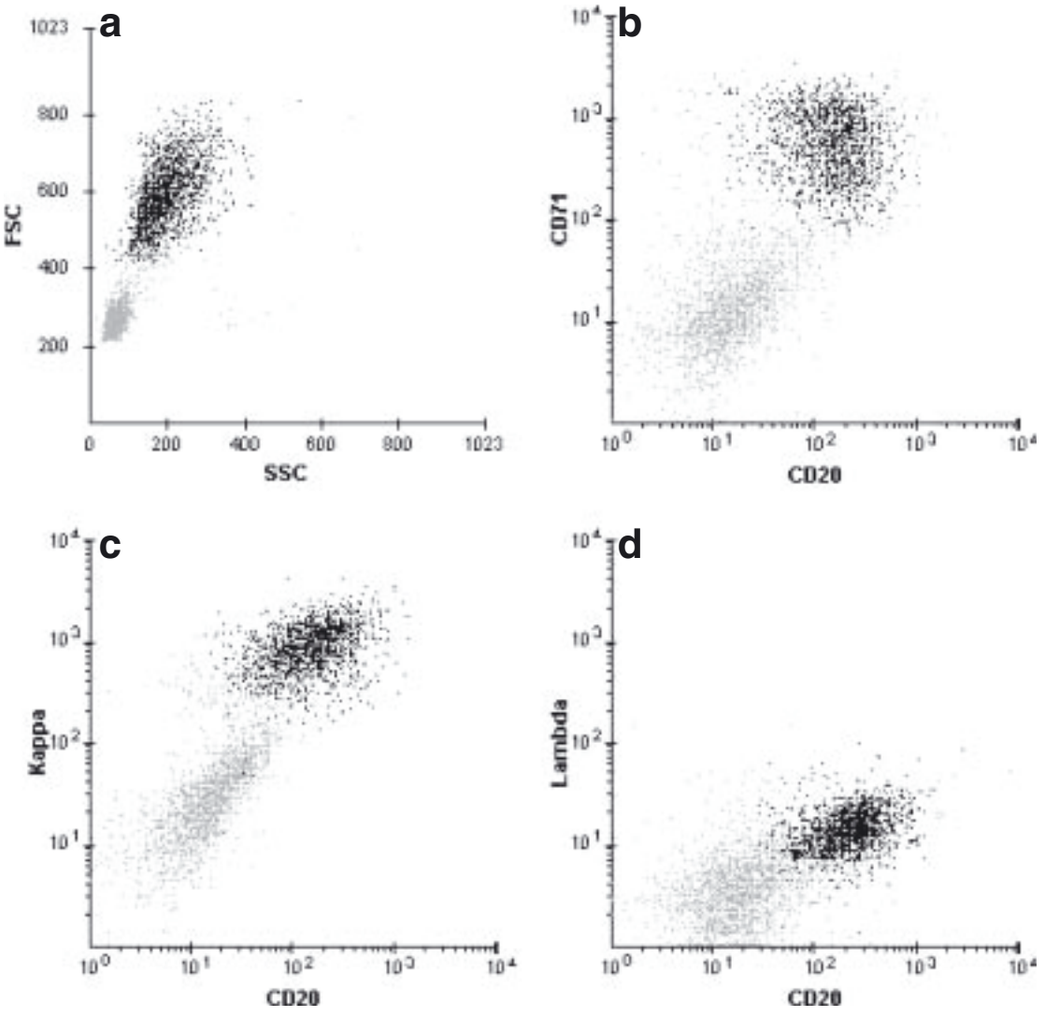

Figure 2.13 Lymph node with high-grade B-NHL. (a) Two distinct cell populations differing in cell size (FSC), for which the use of colors can be applied. (b–d) Cells with high FSC (black) are neoplastic B-cells monoclonal for kappa and expressing high levels of CD71. Cells with low FSC (gray) are benign T-cells negative for the markers displayed.

specimen, there is an apparent continuous population with heterogeneous HLA-DR intensity, from negative to extremely bright. On color displays, it can be more easily appreciated that the apparent continuous population is actually composed of several cell populations of approximately the same cell size merging into each other (Plate 1). In other instances, what appears to be two adjacent cell clusters is actually one cell population with bimodal reactivity for a given marker. With appropriate color displays, potential confusion of one versus two populations may be avoided (Plate 2).

Many commercially available FCM software packages assign a hierarchy to the colors selected by the user, the color selected first being on the top of the hierarchy. Where two cell clusters overlap with each other, the one with the higher-ranked color will obscure the other, partly or completely, depending on the degree of overlap. For example, on the SSC/CD45 dot plot, the colors red and light green are assigned to the monocytic and myeloid populations, respectively, with the latter being the higher-ranked color. On another dot plot (e.g.,

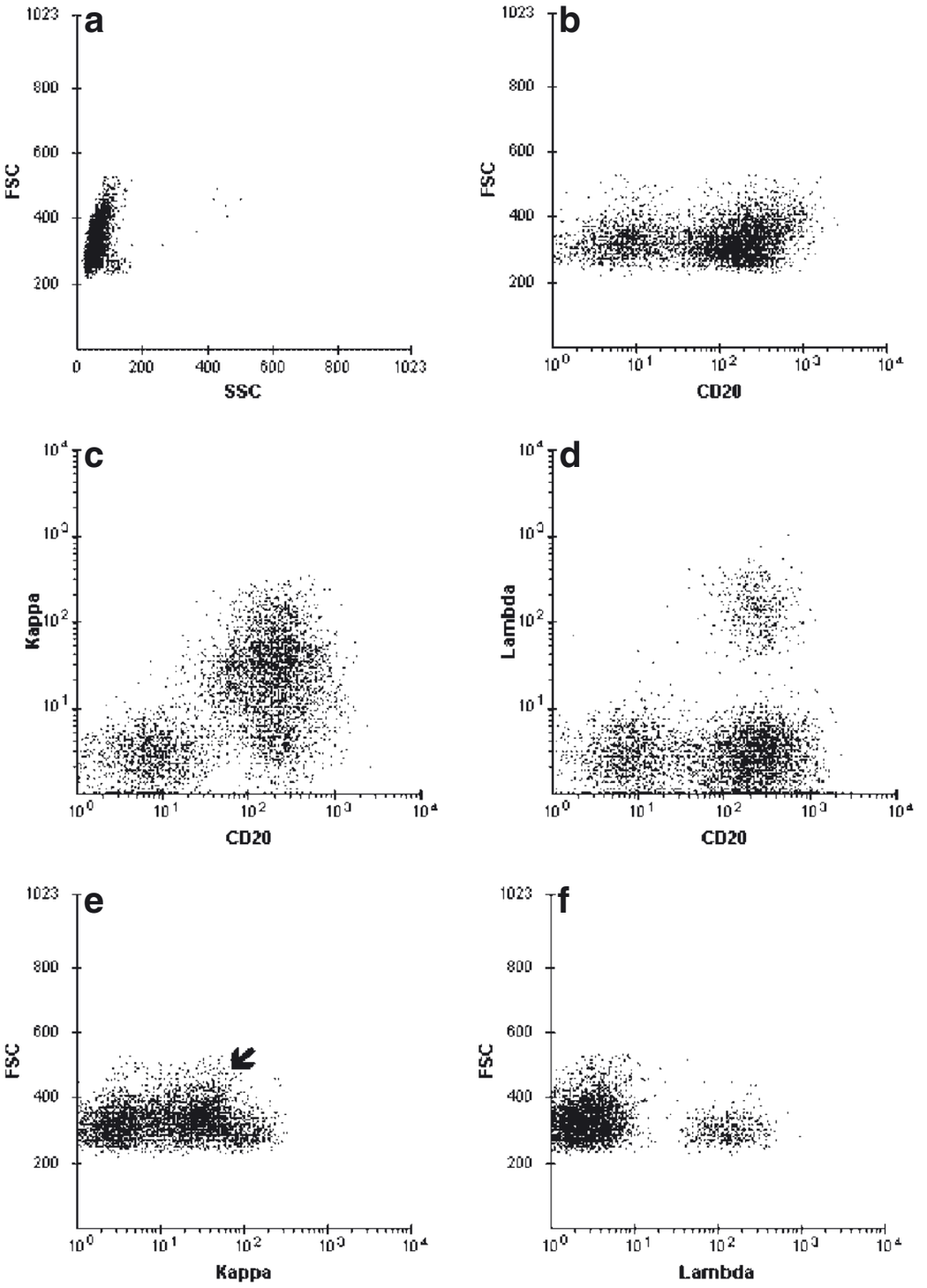

Figure 2.14 Utility of FSC versus fluorescence displays. The lymphoid cells are of similar cell size (a, b) despite their antigenic heterogeneity. (b–d) The benign and neoplastic B-cells share similar intensities of CD20 and CD19 (not shown). It is thus difficult to evaluate monoclonality and quantify the malignant cells on the dual fluorescence dot plots. (e, f) The monoclonal cell cluster (arrow) is more clear-cut on the FSC/kappa (evaluated together with FSC/lambda) display.

CD33/CD34), these two populations largely overlap each other. Despite the fact that red is a bold and darker color than light green, very few, if any, red signals can be seen through the lighter green cluster (Plate 3). The effect is equivalent to that caused by an opaque light green color. In such instances, there is no advantage compared with a monochrome (black-and-white) display. This hierarchical "opacity" is the main limitation to the usefulness of the color display. In some cases, the use of colors may be distracting, causing subtle findings to be paradoxically missed. In the authors' opinion, it is important to be versed in inspecting the immunophenotyping data irrespective of whether the graphics are in color or black-and-white.

2.10 Approach to DNA data analysis

The purpose of DNA analysis is to determine the DNA content (i.e., the ploidy level) of the cells of interest and their growth rate (i.e., the relative proportion of cells in each phase of the cell cycle). The cell cycle can be compartmentalized based on the amount of DNA in the nucleus at a given time in the cycle. Cells with the 2N amount of DNA are either noncycling (G_0 phase) or in the presynthetic growth (G_1) phase of the cell cycle, during which RNA and some proteins are accumulated. This is followed by the synthetic (S) phase during which DNA is being replicated. Cells in the S-phase have an intermediate amount of DNA between 2N and 4N. After DNA duplication, the cells enter the postsynthetic (G_2) growth phase and, finally, undergo mitosis (M). By FCM, cells in the G_2- and M-phases are considered together, as they both have a 4N amount of DNA.

DNA fluorescence has a relatively limited biological dynamic range (typically, fourfold to eightfold). The measurements are therefore made using linear amplification instead of the logarithmic scale used for antigenic fluorescence. The approach to DNA analysis is to first inspect the dot plot, FSC versus fluorescence of the DNA dye, correlating the total DNA content and cell cycle measurements with the relative cell size (Figure 2.15a). The FSC/DNA fluorescence dot plot is also useful for detecting small aneuploid population(s), which may be missed on the single parameter DNA histogram, and evaluating the presence of debris and

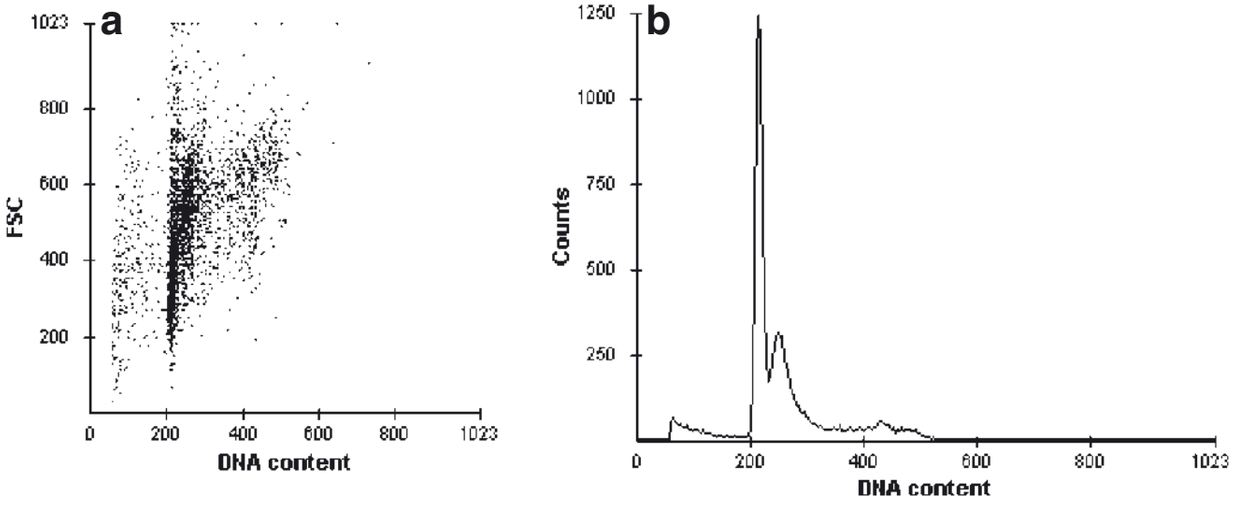

Figure 2.15 Lymph node with high-grade B-NHL. Cells were fixed in ethanol, treated with RNAse and stained with PI. Doublets were excluded. (a) The aneuploid cells are larger than the diploid cells. Most of the S-phase signals are associated with the aneuploid cells. (b) The same data shown on the DNA content histogram. Using a diploid control (not shown) it was established that the left peak represents diploid cells. The tumor is aneuploid (DI: 1.16) and highly proliferative (S% 23.9).

aggregates. The conventional approaches to correct for doublets and aggregates have been based primarily on the altered pulse shape produced by the doublets or clumps when illuminated by a focused laser beam. For instance, using the gating strategy of integrated versus peak DNA fluorescence, doublets of G_0/G_1 cells can be eliminated because they produce an integrated fluorescence equivalent to a G_2/M cell, but a peak fluorescence equal to a G_0/G_1 cell. This exclusion technique does not eliminate the type of doublet caused by two G_0/G_1 cells passing through the laser side-by-side, however. An alternative approach to this electronic exclusion is to use commercially available DNA analysis software whereby mathematical algorithms can be applied to subtract doublets based solely on the DNA content distribution.

2.10.1 DNA ploidy

The ploidy status of the cells of interest is determined by the position of its G_0/G_1 peak relative to the G_0/G_1 peak of diploid cells. By convention, the aneuploid peak must be separable from the diploid peak (i.e., the histogram is bimodal, with a trough between the diploid and aneuploid populations) (Figure 2.15). The DNA index, which reflects the DNA content in the aneuploid population, is the ratio of the peak fluorescence of the aneuploid G_0/G_1 peak to that of the diploid G_0/G_1 cells. The resolution of two cell populations depends on the CV of the DNA analysis and the percentage of aneuploid cells in the sample. The closer to diploid the aneuploid cells are and the lower the proportion of aneuploid cells, the lower the CV must be to achieve separation. Most lymphomas are diploid or near diploid; frank aneuploidy and polyploidy are less frequent (Figure 2.16). Therefore, if the specimen contains a large nonneoplastic population, this diploid component may obscure the near-diploid neoplastic cells on the single parameter DNA histogram and interfere with calculations of the tumor cell S-phase. Prior to the replacement of PI by DRAQ5, the solution to this problem was to perform DNA testing paired with an appropriate FITC-conjugated antibody (e.g., CD20) that would help to delineate the abnormal population (Figure 2.17). With the routine combination of DRAQ5 and two other dyes for surface antigens, measurements of the DNA content can be directly obtained on the gated tumor cells (Figure 2.18) or, where applicable, on a subpopulation thereof. In addition, the implementation of DRAQ5 has also eliminated the technical limitations associated with PI, including the artifact-induced aneuploidy (e.g., ethanol-fixed, PI-stained whole blood or bone marrow may produce extra peaks on the DNA histograms because of the presence of granulocytes).

In ALL, aneuploidy (especially in combination with TdT) is a useful marker for detecting low-level involvement of a sanctuary site (e.g., CSF), as well as for monitoring residual disease in a posttreatment hypocellular marrow or impending relapse in a regenerative marrow (Figure 2.19). In addition, DNA ploidy may be of prognostic significance in ALL. Data on pediatric ALL have revealed that a DNA index (DI) greater than 1.16, which corresponds with the presence of more than 53 chromosomes, is associated with longer remission. Although there is a good correlation between FCM aneuploidy and cytogenetic aneuploidy, karyotypic abnormalities (e.g., small deletions, balanced translocations) that result in a pseudodiploid karyotype are not detectable by flow cytometry.

2.10.2 S-phase

Calculation of the cell cycle phases requires the deconvolution of the DNA histogram into three areas (G_0/G_1, S, and G_2/M) and integration of each individual area. The main difficulty in this process is to separate cells in early S-phase from those in G_0/G_1, and cells in late S-phase from those in G_2/M. Various computing methods have been applied to calculate the frac-

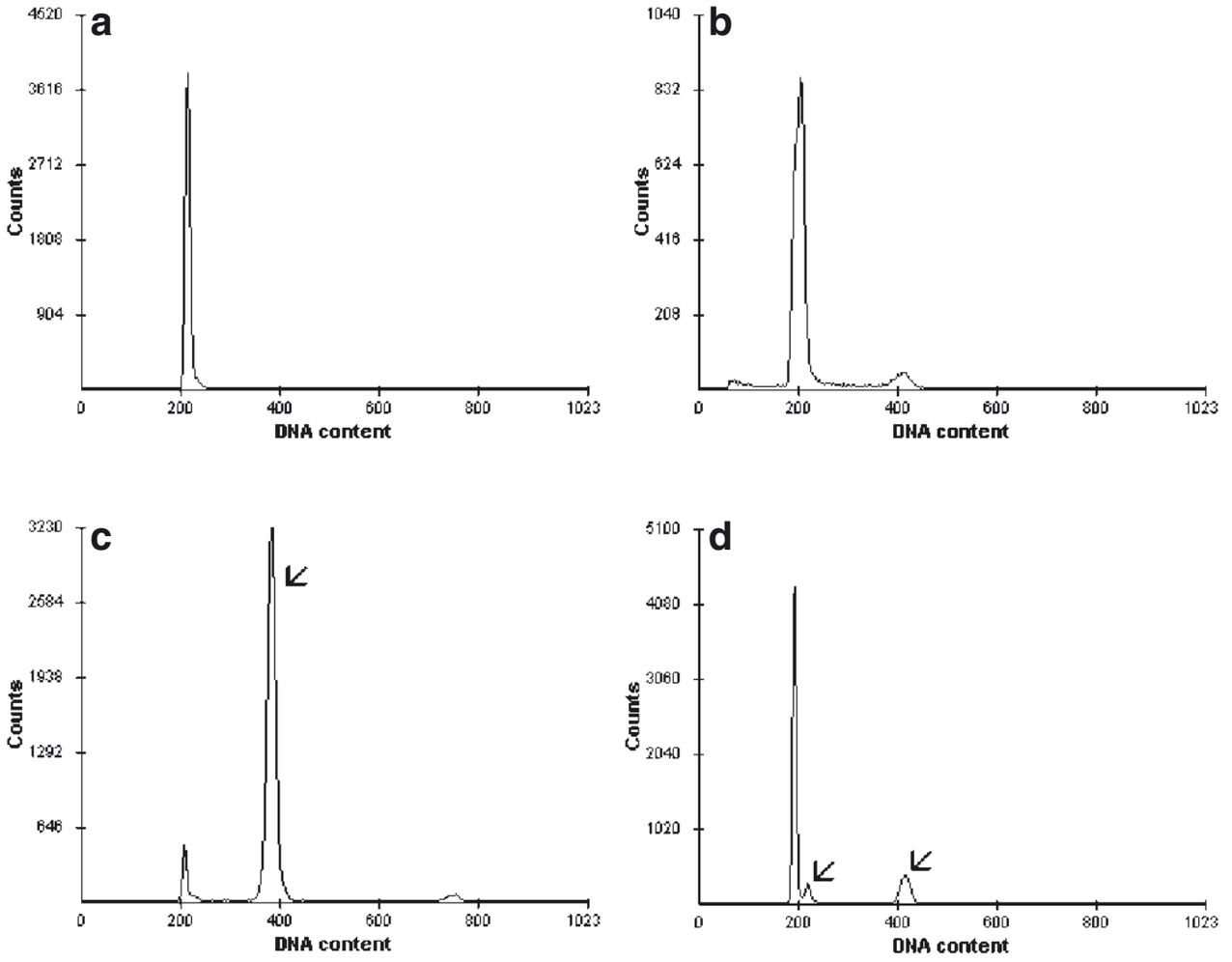

Figure 2.16 Examples of DNA histograms on different lymphomas. (a) FCC II lymphoma: Diploid and low S-phase fraction (S% 2). (b) PTCL: Diploid, S-phase fraction in the intermediate range (S% 10). (c) PTCL: The tumor (arrow) is near tetraploid (DI: 1.84) with an S-phase fraction of 6%. (d) FCC II lymphoma: The tumor (arrows) is polyploid and the S-phase low (DI: 1.1 and 2.1, S% 2).

tion of cells in each phase of the cell cycle. Commercially available programs can be applied to complex histograms (e.g., the presence of debris or overlapping peaks) or those with very high proliferative fractions. For discrete populations with a rather evenly distributed S-phase, low content of debris, and low to intermediate S-phase fractions (<15%), a simple rectangle method for S-phase calculation is suitable to most cases of malignant lymphoma.

With PI-based DNA analysis (i.e., prior to the DRAQ5 era), the presence of normal cells in the sample can affect the cell cycle phase calculations on the neoplastic cells, and therefore the calculations need to be corrected by subtracting the normal cells from the G_0/G_1 peak. If the abnormal cells cannot be identified by a specific feature such as higher FSC, one can assume that normal cells, in specimens other than the bone marrow, are mostly quiescent and do not contribute significantly to the cycling pool. This assumption can be applied irrespective of how close the DNA peak of the neoplastic cells is to that of the normal cells.

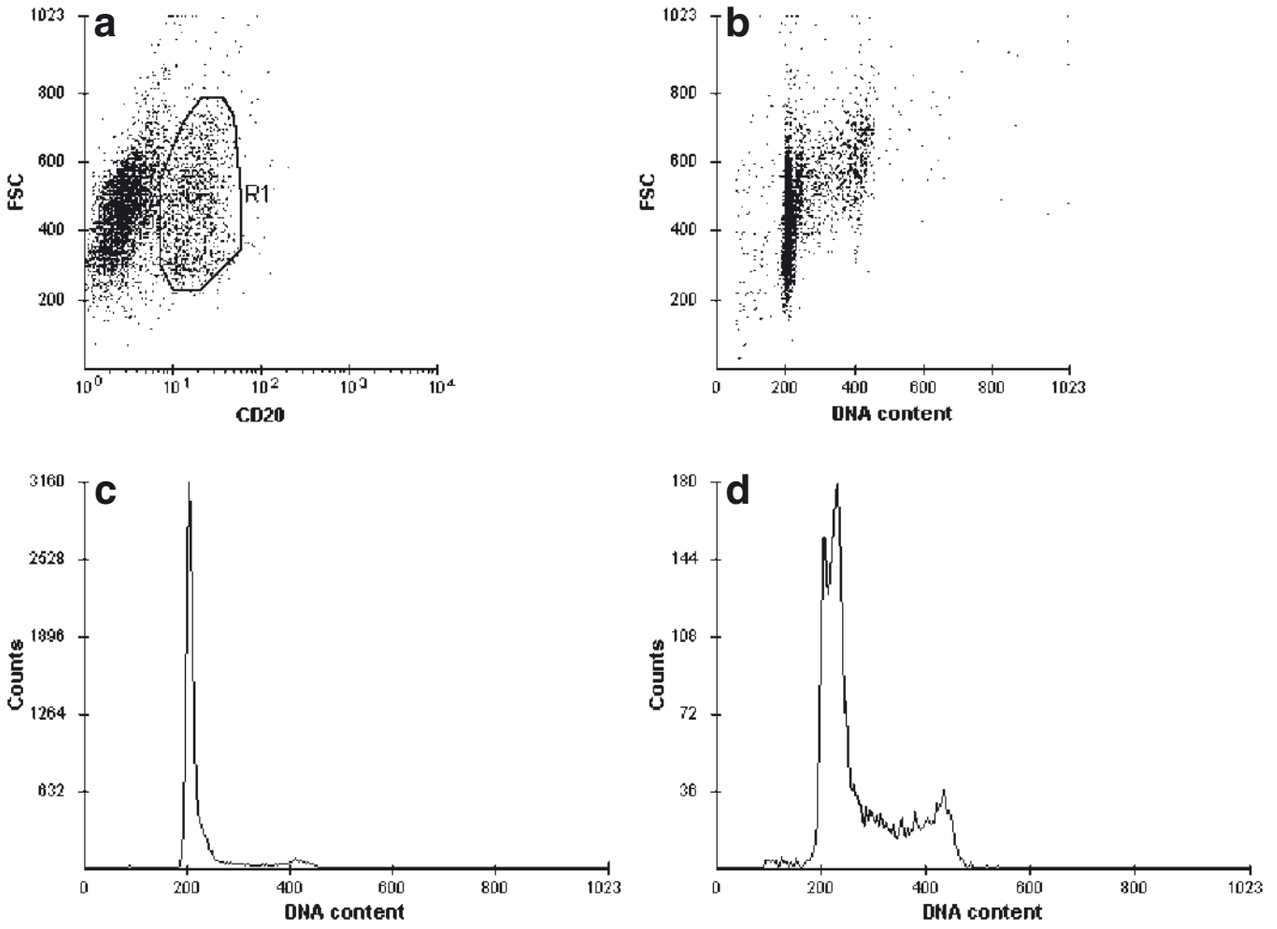

Figure 2.17 Combined analysis of light scatter, CD20 and DNA content to detect and characterize the presence of high-grade B-cell NHL in the peripheral blood. (a) The neoplastic B-cells (R1) are of heterogeneous cell size and comprise 16% of the total cell population. (b) DNA analysis on all cells in the sample. The S-phase signals are associated with large cells. The near diploid component is more discernible on the FSC/DNA dot plot than on the single parameter DNA histogram (c). (d) DNA analysis on $CD20^+$ cells only (R1 gate): The tumor is near diploid (DI: 1.1) and extremely proliferative. The tumor-associated S-phase fraction (S% 54) is much higher than that determined on the entire sample (c).

The preferred approach, especially if the normal and neoplastic populations closely overlap, is multiparameter DNA–antigen analysis whereby an uncontaminated tumor cell cycle fraction can be calculated (Figures 2.17, 2.18, and 2.20). This is the method of choice when the aneuploid cells are the minor component and/or the normal cells are bone marrow hematopoietic precursors, whereby the S and G_2/M signals from the tumor cells on the single parameter DNA histogram are obscured by the "noise" from the larger normal component.

In lymphomas, the S-phase fraction has been proven to correlate well with the biological grade (i.e., clinical course) of the disease. Highly proliferative lymphomas confer a shorter remission duration and poorer overall survival than lymphomas with a low S-phase. On the other hand, the incidence of clinical "complete remission" is higher in lymphomas with a high S-phase fraction because highly proliferative tumors are more amenable to aggressive therapy. Thus, the S-phase fraction is extremely useful for grading lymphomas and assessing progression/transformation from low-grade to aggressive lymphoma, irrespective of the histological classification scheme. From studies correlating the S-phase fraction in lymphomas with the clinical

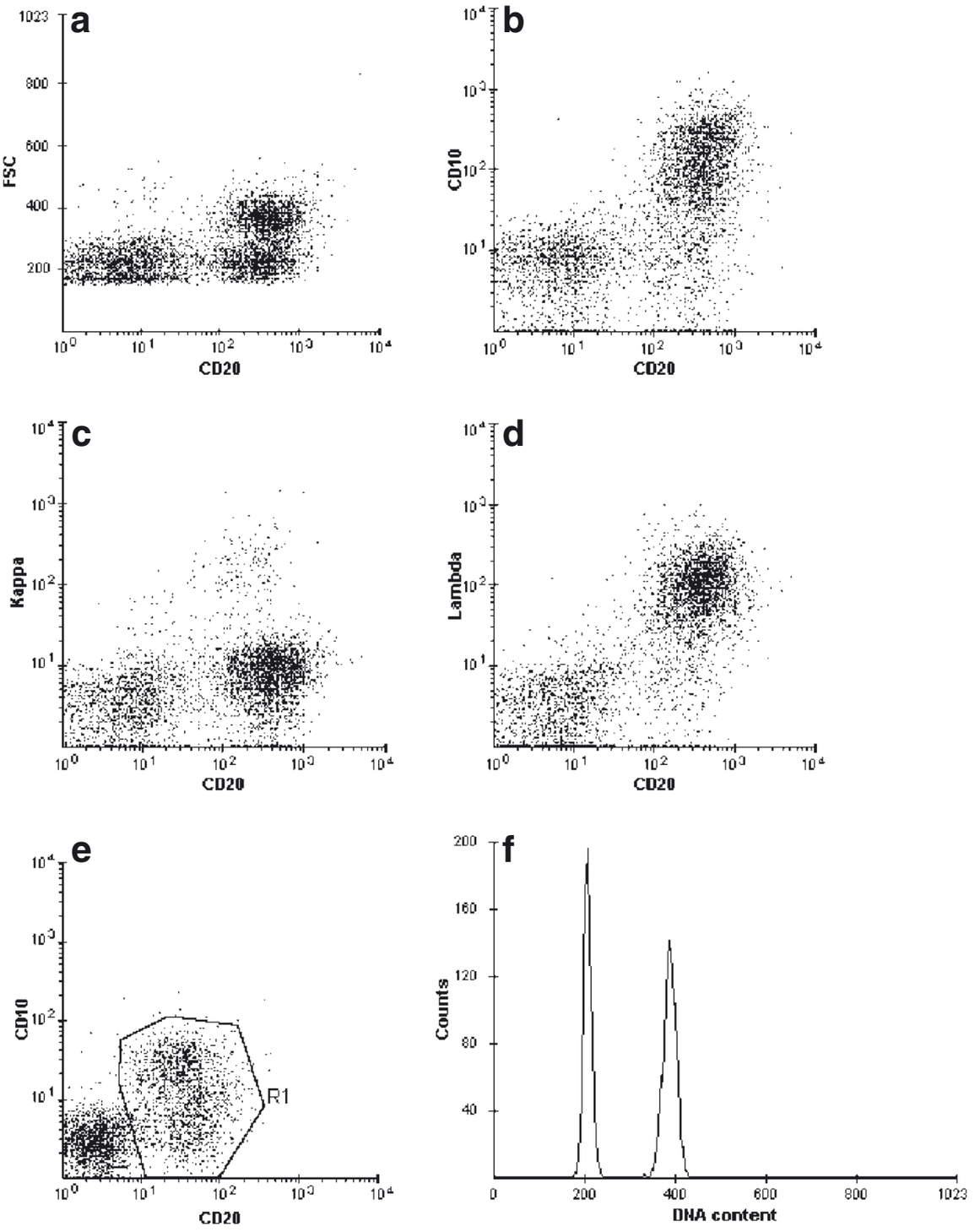

Figure 2.18 Lymph node with follicular lymphoma. The tumor cells are $CD10^+$ (b) and monoclonal for lambda (c, d) with a bimodal cell size distribution (a). (e) DRAQ5 tube: Gated on the $CD10^+$ $CD20^+$ cells for DNA analysis. From this tube, the intensities of the surface antigens (CD10, CD20) are invariably artifactually reduced. (f) The lymphoma is near tetraploid (DI: 1.9) with a low S-phase (1%).

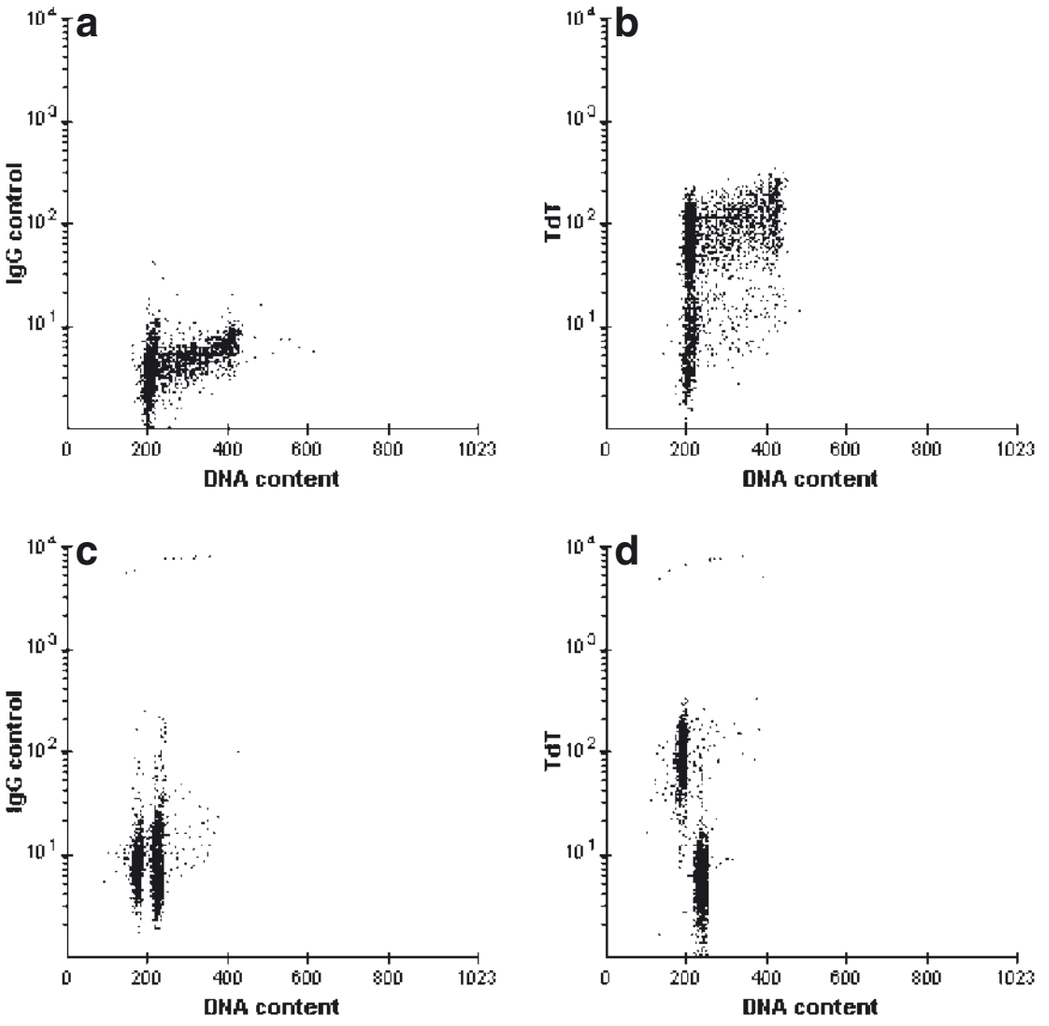

Figure 2.19 Combined analysis of TdT and DNA content applied to two different cases of precursor B-ALL with appropriate controls (a, c) included. (b) Blasts in the first case are TdT positive and diploid. The proliferative fraction is high. (d) Blasts in the second case are TdT positive and hypodiploid (DI: 0.77). The S-phase fraction is low.

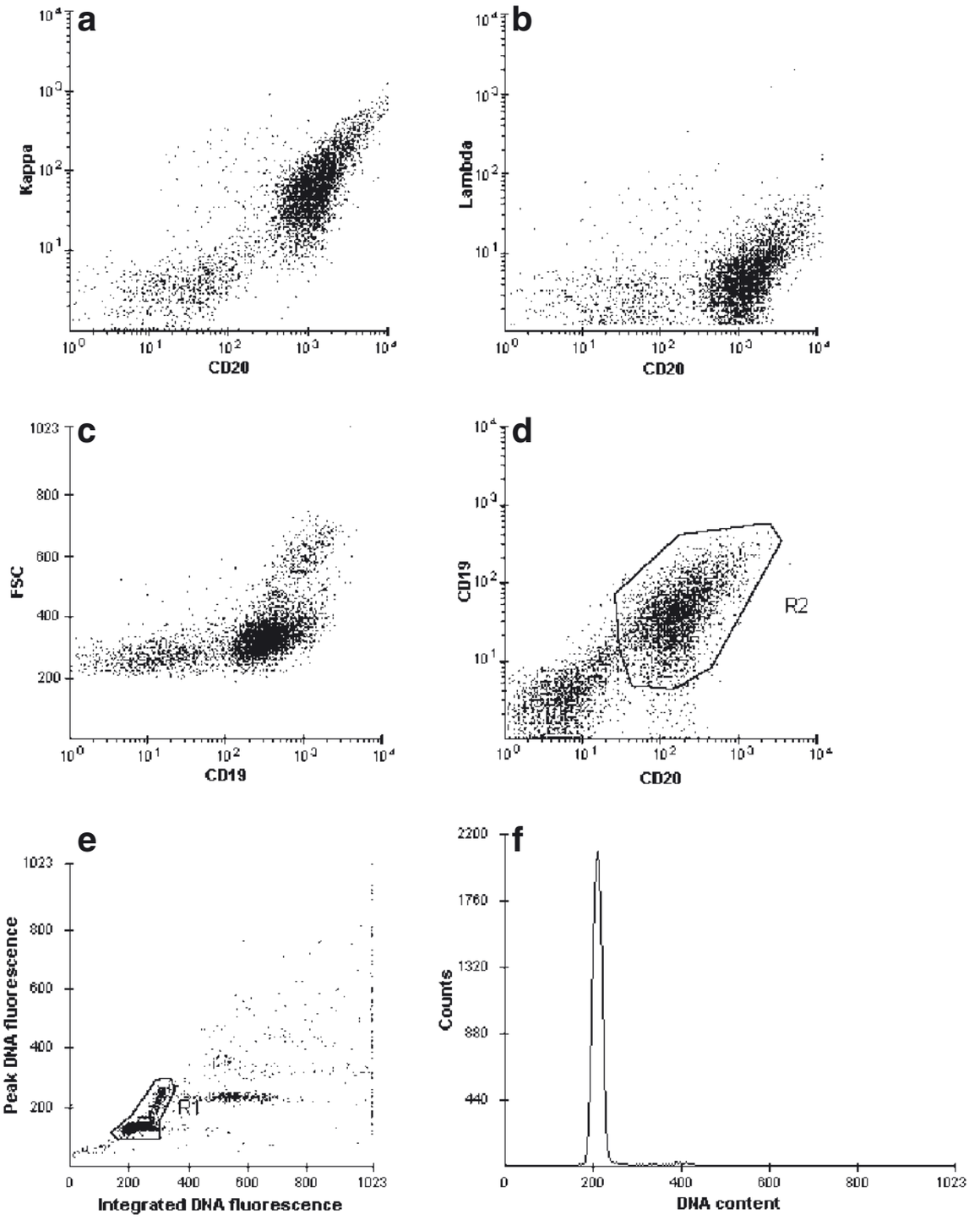

Figure 2.20 B-cell lymphoma, intermediate grade. (a–c) The neoplastic cells display distinct expression of CD19, CD20 and kappa. The cell size ranges from small to medium. Higher FSC signals (beyond 500) are artifacts. (d–f) DRAQ5 tube: DNA analysis is gated on CD19/CD20 cells (d). Doublets are excluded (e). The tumor is diploid (DI: 1) with an intermediate S-phase of 8% (f).

survival and/or relapse-free interval, an S-phase threshold can be derived to establish the grading and prognostic categories. The accepted S-phase threshold for lymphoma grading is 5%. Because FCM parameters, similar to any other laboratory data, contain some margin of error, it is preferable to create three rather than two prognostic groups. Low-grade lymphomas invariably demonstrate proliferative fractions well below the 5% level. The S-phase fraction in more aggressive disease is scattered over a wide range, however. In this group, high-grade lymphomas such as Burkitt lymphoma or B-cell lymphoma with "plasmablastic" differentiation (also referred to as immunoblastic lymphoma) have S-phases in the range of 20% and beyond. In between the two extremes of low- and high-grade is the category of intermediate-grade lymphomas in which the S-phase fraction usually falls between 5% (or the vicinity thereof) and 15%.