Flow Cytometric Analysis of Leukemia and Lymphoma - The Basics

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Deutsche Version

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Summary

Flow cytometry is an indispensable tool for diagnosis and monitoring of leukemia and lymphoma. While application of flow cytometry in this field may be complex and require a lot of experience, it is based on rather simple principles. With the help of many figures this page is supposed to clearly explain these principles.

I. Why does the doctor request a flow cytometric analysis?

II. How can we detect that there is an abnormal cell population?

III. Flow cytometric analysis of mature B-cell neoplasms

IV. Flow cytometric analysis of mature T-cell or NK-cell neoplasms

V. Flow cytometric analysis of acute leukemia

Other related material on this website (in GERMAN, PDF-Files)

- Review Hairy cell leukemia (German)
  Size: 270 Kbytes.
- Application of flow cytometry in the diagnosis a leukemia and lymphoma (German)
  Size: 153 Kbytes.
- Application and limitations of flow cytometric analysis of leukemia and lymphoma (German)
  Size: 507 Kbytes.

I. Why does the doctor request a flow cytometric analysis?

TOP

Of course, there are a lot of indications for a flow cytometric analysis in hematology. However, in principle, we can define 3 main indications.

- **What's that?**
  The doctor discovers something, however, he needs further information about what he sees?
  - E.g., a patient shows an increase of lymphocytes in his blood. The reason for this may

be a leukemia or it may just be a reaction to a viral infection. With flow cytometry this important discrimination is rather easy.
- Another example: in the microscope you definitely spot blasts, you are sure that the patient has an acute leukemia. But what kind. It may be an acute lymphocytic leukemia or an acute myeloid leukemia. This distinction is important for therapy and for prognosis. Flow cytometry usually can give the answer.
- Sometimes flow cytometry helps to define the leukemia subgroup. For example the so called M7, the megakaryoblastic leukemia.
  Flow cytometry also helps to define lymphoma subgroups, for example the distinction between CLL, hairy cell leukemia or other subgroups.

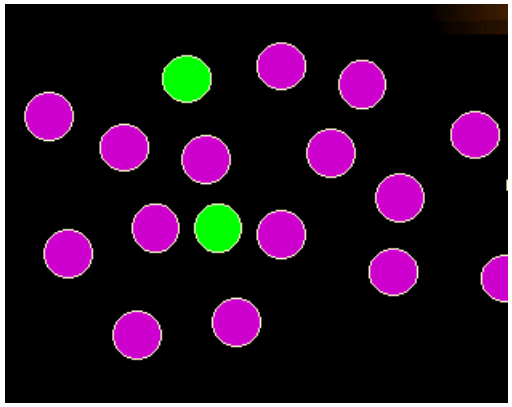
- **Is there anything abnormal in the sample?**
  There is no particular suspicious cell population but the patient's symptoms suggest leukemia or lymphoma as a possible cause.
  Such cases are not easy and often unrewarding. Sometimes it is like searching for the needle in the haystack. And sometimes we are even searching the wrong haystack, when blood is sent to the lab but the lymphoma cells are only present in the bone marrow or in the lymph node. The referring doctor should be aware of these limitations. And he should be aware of what can and what can never be detected by flow cytometry in order to correctly interpret a negative result.

- **Is there still something?**
  When monitoring leukemia and lymphoma we try to find the malignant cells that were detected originally.
  That's an easy task when the malignant cells can be clearly separated from the normal cells. In these cases even very small populations (e.g. 0.1% or less) of malignant cells can be detected.
  Monitoring is much more difficult when the malignant cells are very similar to normal cells. Then, even larger population of malignant cells (e.g. 1 or 2%) may remain undetected. In some cases of T-cell lymphoma even larger population may be unnoticed.

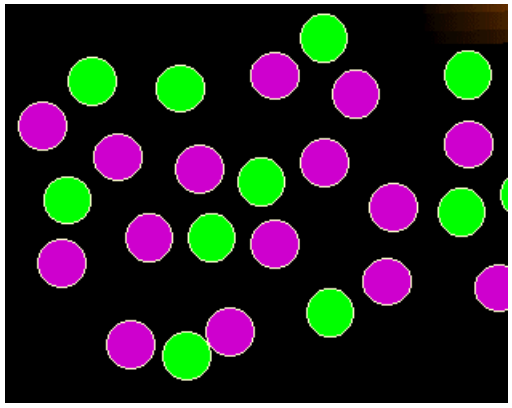
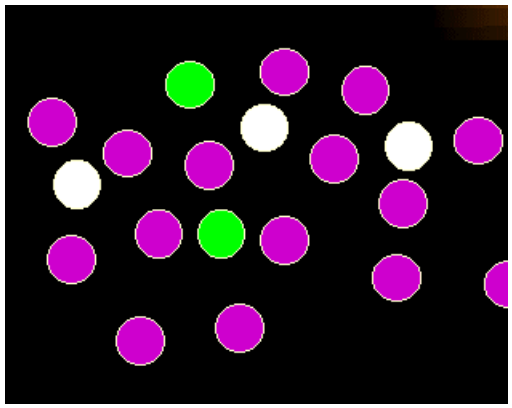
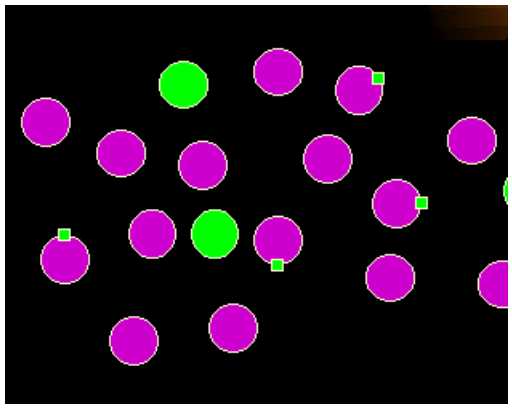
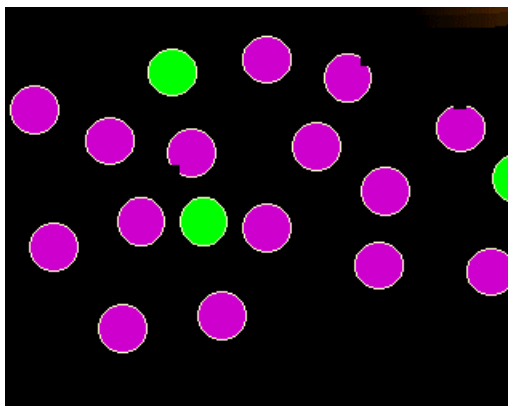
II. How can we find an abnormal cell population?

TOP

While in some cases the detection of abnormality may be very complex, the underlying principles presented in the following figures are rather simple.



Normal finding
Let's assume these are the normal findings. A dominating population of green "cells" and a few green cells.

	**What's wrong in this picture?** You will notice immediately: too many green cells. In other words: **a disproportionate increase of one cell population**. As simple as this may sound, this kind of abnormality is an important sign for the diagnosis of leukemia and lymphoma.
	**What's wrong in this picture?** There is a new cell population, the white cells. They were not present before. **The appearance of cells not normally present** may be another another sign of leukemia or lymphoma.
	**What's wrong here?** There is no disproportionate increase of any population, there are no cells that should not be there but the magenta cells look different. They have something unusual on their surface. The **expression of abnormal antigens** is another important sign for malignancy.
	**Finally, what's wrong here?** The bite-like deficiency in the magenta cells is supposed to symbolize the loss of an antigen. **Loss of an antigen normally present** is another sign for malignancy**.**

That's it. As you can see, there are only a few principles to detect abnormal cells. Application of these principles in many different ways helps to distinguish normal from abnormal cells.

III. Flow cytometric analysis of mature B-cell neoplasms

Preface
This chapter deals with malignant proliferations of mature B-cells (mature B-Non-Hodgkin-Lymphoma, mature B-cell leukemia).
The distinction between lymphoma (disease present primarily in lymph nodes or lymphatic organ/tissue) and leukemia (massive presence of malignant cells in blood or bone marrow) is arbitrary.

Examples of such diseases are chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL), prolymphocytic leukemia (PLL) as well as immunocytoma (IC), mantle cell lymphoma (MCL), follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL).

Why do we start with these diseases? Because flow cytometry plays a big role for their diagnosis and classification.

Chapters:

- The steps towards diagnosis
- The search for monoclonal B-cells
- The search for B-cells with abnormal antigen expression
- Defining antigen expression (=the immunophenotype)
- Diagnosis

The steps towards diagnosis of mature B-cell neoplasms

- **Finding the abnormal B-cell population**
  (Are there abnormal, malignant B-cells at all?)
  - **Look for monoclonal B-cells** (B-cells that are all originating from one cell, as malignant cells do)
  - **Look for B-cells with abnormal expression of antigens**
- **Defining the immunophenotype (the pattern of antigen expression)** of the abnormal B-cell population (which antigens do the malignant cells carry on their surface [and sometimes in their cytoplasm]).
- **Diagnosis**
  Sometimes flow cytometry can give the exact diagnosis, sometimes it helps to narrow down the possible diagnoses.

Note: these steps are not necessarily analytical steps, they are rather a logical concept to approach a diagnosis.

1. The search for monoclonal B-cells

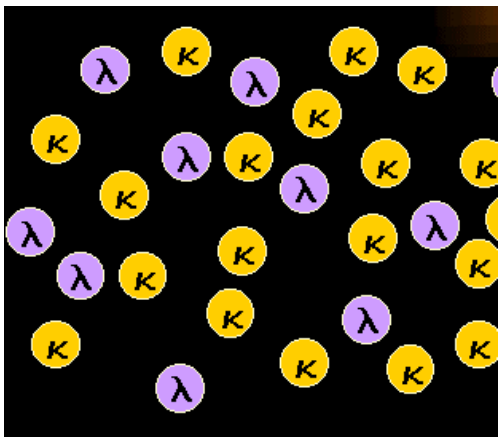
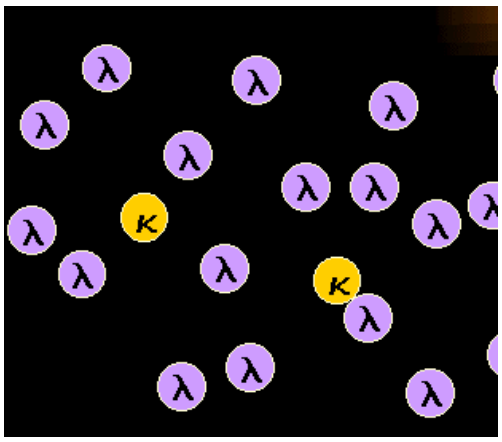
What are monoclonal B-cells?
A B-cell clone is a group of identical B-cells, originating from one cell, as is the case in B-cell leukemia or lymphoma.

Why do we look for monoclonal B-cells?
Monoclonal B-cells are the single most important flow cytometric finding for the diagnosis of a B-cell malignancy. If you do NOT find monoclonal B-cells (despite having looked carefully and in the right place) a B-cell lymphoma or leukemia is very unlikely. Finding monoclonal B-cells and corresponding symptoms in the patient makes a B-cell malignancy very likely.

Are there monoclonal B-cell populations in the absence of B-cell malignancy?
Yes, using methods as sensitive as flow cytometry, it is not uncommon to find small monoclonal

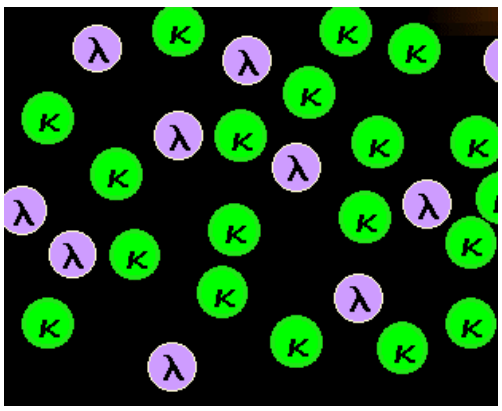
population in patients without any clinical sign of a B-cell malignancy. In some cases this is supposedly a very early stage of a B-cell leukemia or lymphoma. However, there is little data in the literature about the likelihood of progression of the monoclonal population into a B-cell malignancy.

How can we find a monoclonal B-cell population using flow cytometry?
With some cells it is very difficult to establish monoclonality, with B-cells, luckily, it is relatively easy. The following figures should explain why:

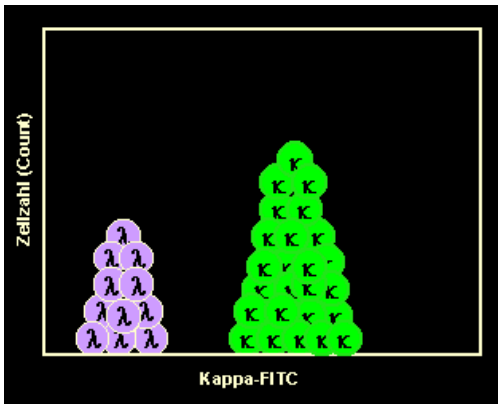
<table>
<tr><td>  </td><td>Normal, polyclonal B-cells are a mixture of kappa-B-cells and lambda-B-cells.
Our B-cells have a special feature letting us detect their clonality easily: A B-cell carries either kappa- or lambda-light chains on its surface. And normal polyclonal B-cells are a mixture of kappa-B-cells and lambda B-cells as can be seen in the left-hand figure.</td></tr>
<tr><td>  </td><td>Monoclonal mature B-cells are either kappa or lambda.
If a malignant B-cell clone proliferates this will result in a B-cell population consisting of either only kappa- or only lambda-B-cells. The latter case (i.e. lambda-monoclonal B-cells) is symbolized in the left-hand figure.
Note: in rare cases we find no light chain expressed on the B-cell surface, even in a mature B-cell neoplasm. This makes the diagnosis a little bit more difficult.</td></tr>
</table>

Thus we see that monoclonal B-cells are no mixture of kappa- and lambda-B-cells like polyclonal B-cells, monoclonal B-cells show a gross preponderance of either kappa- or lambda B-cells.

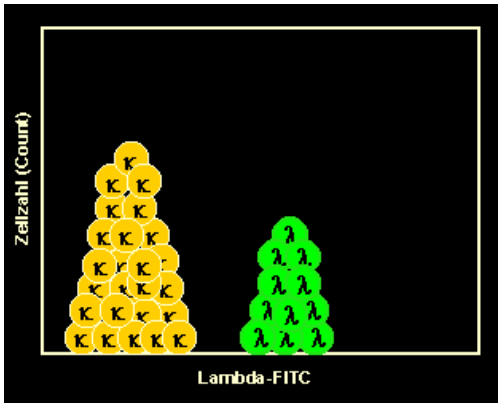
But how can we measure this by flow cytometry?
First, we stain the light chain (kappa or lambda) with suitable fluorescence-labeled antibodies. Then we run the sample on the flow cytometer:

<table>
<tr><th colspan="2">Assessment of B-cell clonality on a flow cytometer
1. Normal, polyclonal B-cells</th></tr>
<tr><td>  </td><td>Staining of kappa-B-cells
When we use an anti-kappa-FITC* antibody all kappa-B-cells will be stained.
Note: you may notice a slight preponderance of kappa cells. That is normal, one usually has slightly more kappa- than lambda-B-cells.

*FITC: Fluoresceinisothiocyanate, a "green" fluorochrome.</td></tr>
</table>



Running the sample on the flow cytometer
After measuring the sample on the cytometer we present the results in a so called one-parameter histogram. You can see a slightly higher peak representing the kappa-B-cells, which are stained by the antibody, and a smaller peak representing the unstained lambda-B-cells. This is the typical picture of polyclonal B-cells.



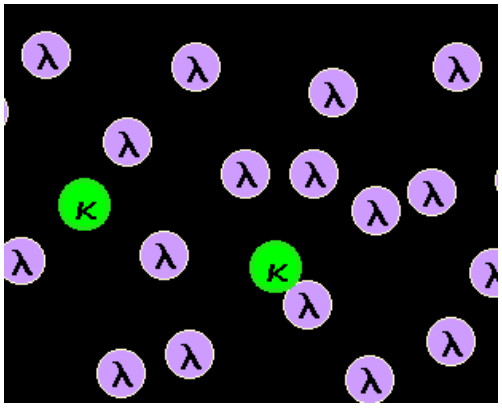
Staining of lambda-B-cells
In another tube we stain the lambda-B-cells using an anti-kappa-FITC antibody. After measurement we encounter a somehow reversed image: A slightly smaller peak representing the lambda-B-cells, which are stained by the antibody, and a larger peak representing the unstained kappa-B-cells.

Note: in these examples we used a FITC-labeled antibody for kappa and lambda. Therefore, staining had to be done in two separate tubes. Instead, one might use a FITC(green)-labeled for kappa and a PE(orange)-labeled antibody for lambda. Then, one might perform staining of both light chains in one tube. Both methods have their pros and cons. In routine clinical practice we do both.

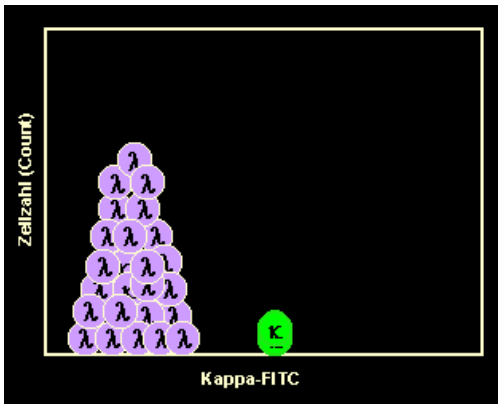
In this first example the B-cell population was polyclonal, suggested by the more or less even distribution of kappa- and lambda-B-cells

The next example looks different:

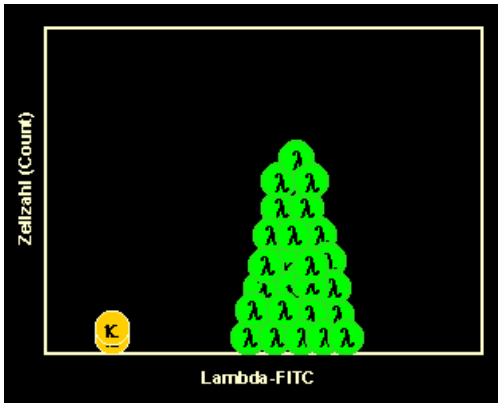
Assessment of B-cell clonality on a flow cytometer
2. Abnormal, monoclonal B-cells



Staining of kappa-B-cells
Only a few cells are stained because the majority of cells are lambda.

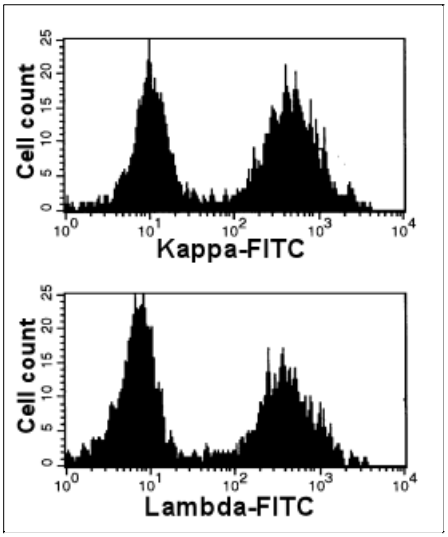


Running the kappa-stained sample on the flow cytometer
That's how the results of the flow cytometric measurement look like: There is nearly no kappa-peak. The unstained B-cells dominate.



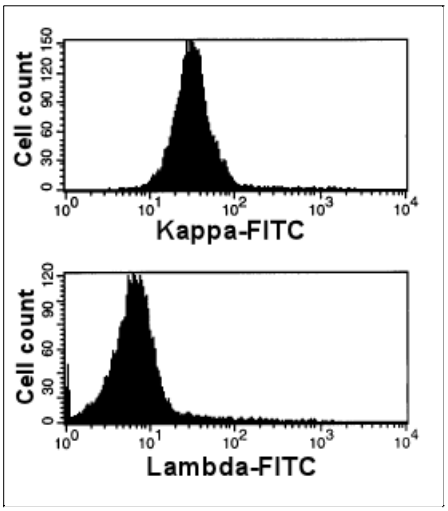
Staining of lambda-B-cells
Accordingly, the stained B-cells dominate after staining with anti-lambda-FITC. This suggests the presence of a monoclonal, lambda-positive (= a lambda-monoclonal) B-cell population which may be leukemia or lymphoma cells.

The figures shown so far were only schematic. In reality the histograms generated after flow cytometric measurement look different. The following figures show normal polyclonal as well as abnormal monoclonal B-cell populations:



Normal, polyclonal B-cells
The two peaks after kappa- and lambda staining suggest an even distribution of kappa- and lambda-B-cells. This suggests a normal polyclonal B-cell population.

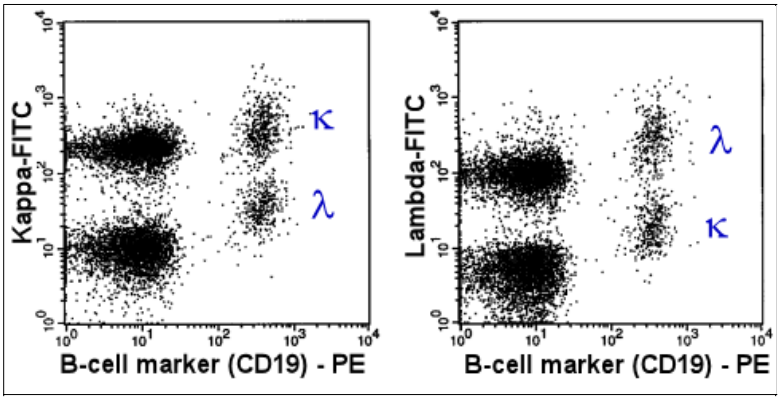
Note (just in case you wondered): these histograms show that the **majority** of B-cells is polyclonal. A very small monoclonal population, however, may remain undetected. See below how to define clonality in a minor B-cell population.



Abnormal, monoclonal B-cells
Upper panel: Here you see only one dominating peak. These cells are all kappa-positive. This is due to the presence of a kappa-clonal B-cell clone. Nearly all of the B-cells are part of the clone.
This is a case of chronic lymphatic leukemia (CLL).
Lower panel: The same cells are stained with anti-lambda-FITC. As expected, they are negative.

Without mentioning it, the figures above did only show the B-cells, because these were our cells of interest. In a normal sample, however, we encounter a number of different cell populations, of course. Not only B-cells. And these cells may stain positive when staining with anti-kappa or anti-lambda antibodies and may, therefore, obscure our results.
Nowadays this is not a big problem: in addition to a FITC-labeled anti-kappa or anti-lambda we stain the cells with an anti-B-cell antibody, e.g. anti-CD19 or anti-CD20.
Then, we can display our results in a so-called two-parameter dot-plot, which may look like this:

Normal, polyclonal B-cells displayed in a 2-parameter dot-plot

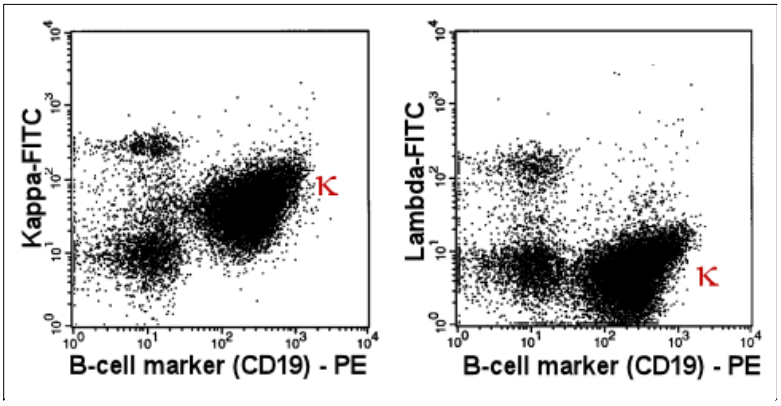


In 2 tubes the B-cells were stained with an PE(orange)-labeled anti-CD19 antibody. Simultaneously, the light chain was stained with a FITC-labeled antibody. Anti-kappa-FITC in the first tube (left panel) and anti-lambda-FITC in the second tube (right panel).

The population designated with the Greek **k** and **l** are the kappa- and lambda-B-cells, respectively. The distribution is more or less even. This suggests a polyclonal B-cell population.
Note for those interested: the CD19-negative populations (on the left side of the dot-plots) are T-cells (lower population) and monocytes (upper population). The monocytes show a strong non specific binding of antibodies (via Fc-receptors) and therefore are strongly positive when staining with anti-light chain or anti-heavy chain antibodies.

The figure above shows the typical picture of a normal polyclonal B-cell population.
Compare this picture to the light chain expression of the malignant cells in a case of chronic lymphocytic Leukemia (below).

Abnormal, monoclonal B-cells displayed in a 2-parameter dot-plot

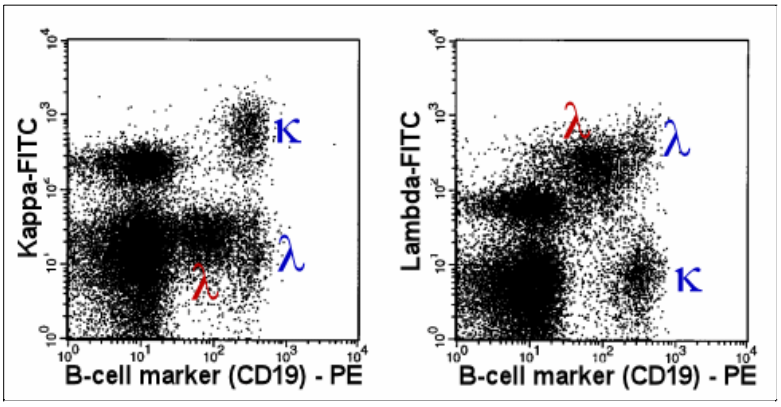


In 2 tubes the B-cells were stained with an PE-labeled anti-CD19 antibody. Simultaneously, the light chain was stained with a FITC-labeled antibody. Anti-kappa-FITC in the first tube (left panel) and anti-lambda-FITC in the second tube (right panel).

The Greek **k** designates the kappa-B-cells. Their extreme preponderance proves the monoclonality of the B-cell population.

Combining light chain staining with staining of other B-cell antigens is not only done to identify the B-cells. It can also help to detect smaller monoclonal B-cell populations.
This is shown in the next example:

Finding smaller monoclonal B-cell population by combined measurement of various B-cell antigens



The lambda-monoclonal B-cells (red Greek **l**), are easy to detect, because these cells show a weaker expression of CD19 than the normal, polyclonal B-cells.

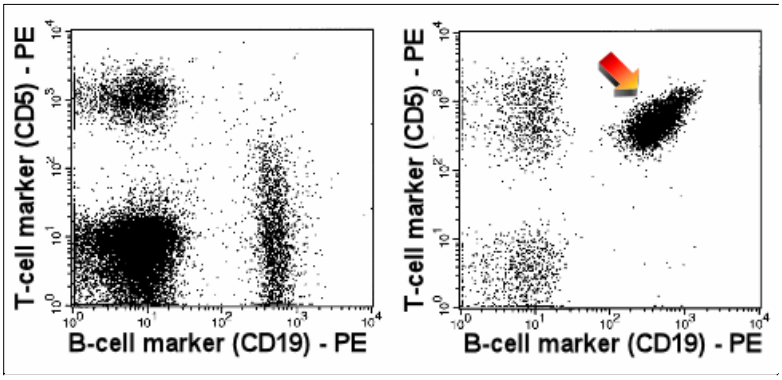
In this case the monoclonal population is rather small. It is not dominating as in the previous case. Therefore, in a one-parameter histogram it would be difficult to detect. In a 2-parameter dot-plot the monoclonal population is easy to detect: its weaker CD19 expression separates the monoclonal cells (red Greek **l**) from the normal polyclonal cells (blue Greek **k** and **l**).

It is not always CD19. Sometimes expression of CD20, CD79b, CD5, CD10 or CD38 helps to discriminate normal from abnormal cells. By combining the discriminating marker with kappa/lambda-staining, monoclonality can be established even in very small B-cell populations.

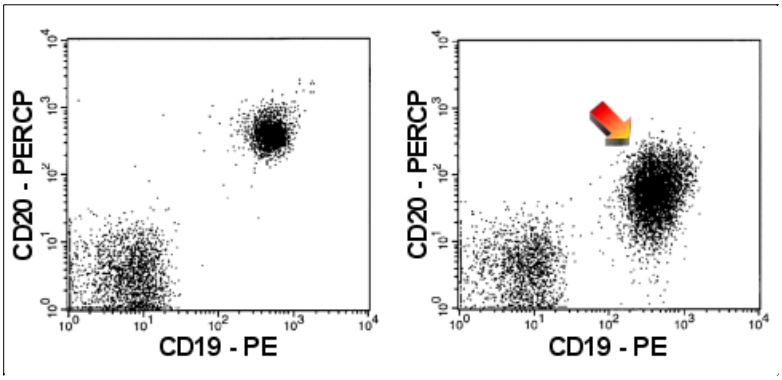
2. The search for B-cells with abnormal antigen expression

TOP

Not as important as the search for monoclonal B-cells, but may help to find abnormal B-cells.



Expression of CD5
The arrow in the right panel points to the abnormal, strong expression of CD5 by B-cells. CD5 expression as strong as this can usually only be found on T-cells. Normal B-cells show no or only a weak expression of CD5 (left-hand panel)



Weak expression of CD20
The B-cells in the right panel show only a weak expression of CD20 (arrow). For comparison: normal CD20 expression in the left-hand panel.

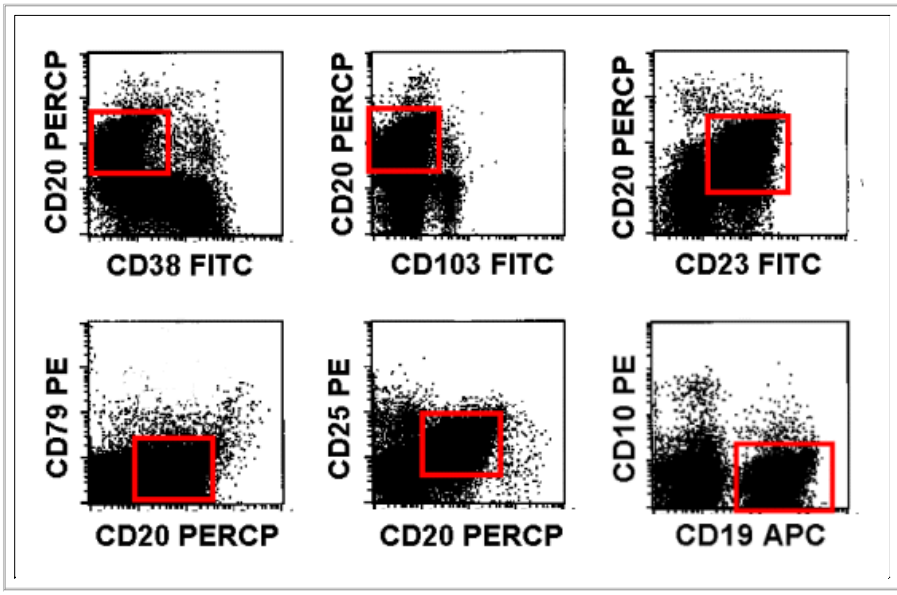
In the right-hand dot-plots you find the B-cells of a patient with CLL (Chronic Lymphocytic Leukemia), which typically show strong expression of CD5 and weak expression of CD20.

Caution: An abnormal antigen expression is generally not a reliable sign of a B-cell malignancy. Especially when only a few B-cells show the "abnormal" expression. In reactive conditions with B-cell activation or even in healthy volunteers it is not uncommon to encounter small B-cell populations with unusual antigen expression. If in doubt about its relevance always try to establish clonality.
When detecting very small populations with abnormal or very unlikely antigen expression one should also consider carry-over as possible reason. Many an unusual population we have found during the last years has turned out just to be a carry over from the previous tube. And some carry over can hardly be avoided, it is introduced by the analyzers aspiration tube.

3. Defining the antigen expression (immunophenotype) of the abnormal B-cells

TOP

Once you have established that there is a monoclonal, most likely malignant population you have to define its immunophenotype, that means you have to check which antigens are expressed on these cells an which are not. Usually that is an easy task. The way we do it, we have stained all these antigens in the first place, so that now we just have to look at the results.



Immunophenotype of abnormal B-cells
The B-cells are CD38neg, CD103neg, CD23pos, CD79neg, CD25pos and CD10neg.

PerCP: Peridinin Chlorophyll Protein
APC: Allophycocyanin

4. Diagnosis of B-NHL

TOP

Preliminary remark
This is a critical point. Can flow cytometry actually establish the diagnosis of a certain B-NHL entity? Not always, strictly speaking not very often. However, flow cytometry can often establish the presence of a clonal B-cell population when other techniques cannot and flow cytometry results usually make certain lymphoma entities extremely likely and others very unlikely. Therefore flow cytometry is an important, integral part of lymphoma diagnosis even in cases where it cannot give a definitive diagnosis.

Procedure
In the last chapter we have defined the immunophenotype of the monoclonal B-cell population. Now we have to find out which lymphoma entity might show such a phenotype.
To give an idea how that works, a table describing the typical immunophenotypes of the most important mature B-cell neoplasms is shown below.

	CLL/SLL	IC	PLL	MCL	HCL	FL
surf.IG	weakly +	+	+	+	+	+
CD5	**+**	- to weakly +	- to weakly +	**+**	-	-
CD10	-	-	-	-	-	**+**
CD11c	- to +	-/+	-/+	-	strongly +	-
CD19	+	+	+	+	+	+
CD20	**weakly +**	+	+	+	+	+
CD22	- to weakly +	+	+/-	+	+	+
CD23	**+**	-	-/+	**-**	-	+/-

CD24	+	+/-	+	+	+	+
CD25	- to mod. +	- to weakly +	-	-	+	-
CD38	- to mod. +	+/-	-		rarely +	
CD43	+	+	+	+	-	-
CD79b	**-**	+	+	+	+	+
CD103	-	-	-	-	**+**	-
FMC7	-	+	+	+	+	+

CLL: Chronic Lymphocytic Leukemia
SLL: Small Lymphocytic Lymphoma
IC: Immunocytoma
PLL: Prolymphocytic Leukemia
MCL: Mantle Cell Lymphoma
HCL: Hairy Cell Leukemia
FL: Follicular Lymphoma

It seems simple: you match the immunophenotype with that described in the table and have the answer. Unfortunately this is not the case.
Primarily because of three problems:

- **A certain immunophenotype may be typical but is by no means obligatory**
  Example: a CLL is typically CD23pos and CD5pos. But it may be CD23neg and it may even be CD5neg. And that goes for practically all antigens. Not one is present in all cases of a certain B-NHL entity, hardly one is reliably never present in that entity.
  Of course one important reason for this is that these entities are not defined by immunophenotype but usually by morphology. That is why there are CD5-negative CLLs. In fact, it is very likely that CD5-positive and CD5-negative CLL are different entities and should probably not be lumped together in one group.

- **The significance of one marker is depending on the expression of the other markers**
  Example: If CD5 is positive, CD23 plays an important role because it helps discriminating CLL from MCL. If CD5 is negative, CD23 expression is not really important.

- **The strength of antigen expression is important**
  With some antigens it is not enough to describe it as positive or negative, it is important how strongly the antigen is expressed. You rarely find this in tables

Mentioning these problems shall make clear that interpretation of flow cytometric findings in hematological malignancy is more difficult than suggested by tables like the one above or flow charts that can sometimes be found to guide flow cytometric diagnosis.

IV. Flow cytometric analysis of mature T- or NK-cell neoplasms

TOP

This chapter deals with malignant proliferations of mature T-cells (T-non-Hodgkin-Lymphoma, T-NHL) and NK-cells. The distinction between lymphoma (disease present primarily in lymph nodes or lymphatic organ/tissue) and leukemia (massive presence of malignant cells in blood or bone marrow) is arbitrary.
Examples are the T-PLL (T-Prolymphocytic Leukemia), the T-LGL-(T-Large-Granular-Lymphocyte-leukemia) and the NK-cell-leukemia.

The role of flow cytometry for the diagnosis of these diseases is not as important as for B-NHL. Nevertheless, in many cases flow cytometry can find the malignant T-cells or NK-cells and can guide the workup appropriately.

Chapters:

- The steps towards diagnosis
- The search for T-cells with abnormal antigen expression
- The (frustrating) search for monoclonal T-cells
- Defining the antigen expression (immunophenotype)
- Diagnosis

The steps towards diagnosis of mature malignant T- and NK-cell diseases

- **Finding the abnormal T- or NK-cells**
  (Are there any malignant T- or NK-cells at all?)
  - **Search for T-cells with abnormal antigen expression**
    In contrast to B-cells where the proof of monoclonality is pivotal, with T-cells finding an abnormal antigen expression is more important. Why? Because finding an abnormal antigen expression is usually all flow cytometry can do.
  - **Search for monoclonal T-cells**.
    Unfortunately, it is rather difficult to establish a reliable, easy, routine flow cytometric method for the detection of T-cell clonality. It requires a bundle of antibodies and even then, clonality will not be reliably detected in all cases. With NK-cells routine flow cytometry cannot prove monoclonality at all.

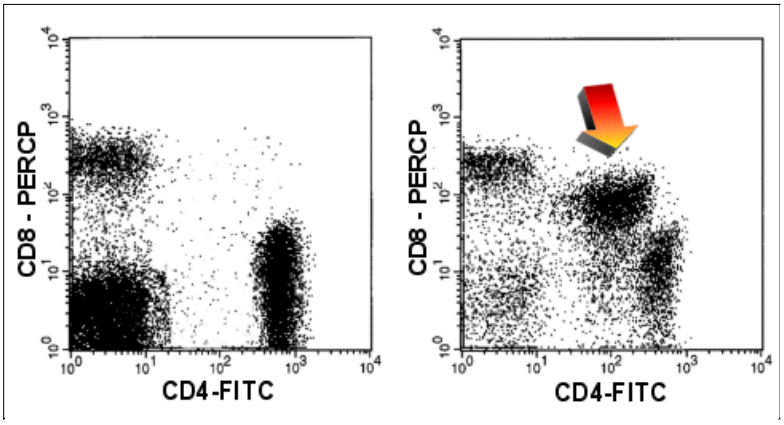
- **Defining the immunophenotype** of the abnormal T- or NK-cell population. As soon as you have clearly defined and discriminated the abnormal population this should not be very difficult.

- **Finding the diagnosis**
  Sometimes flow cytometric results suggest which type of T-NHL is present, in other cases flow cytometry can narrow down the possible entities.

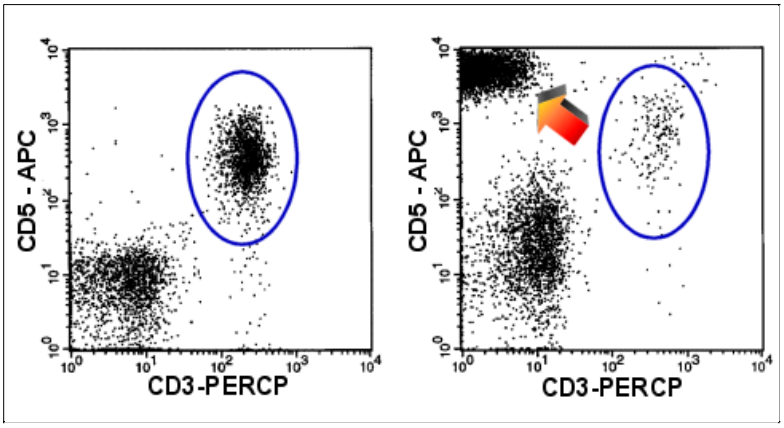
Note: these steps are not necessarily analytical steps, they are rather a logical concept to approach a diagnosis.

1. Search for T-cells with abnormal antigen expression

T-cells with an abnormal antigen expression may be a sign of a T-cell malignancy. Abnormal antigen expression may be the loss of an antigen, expression of an antigen not usually present or the over- or under-expression of an antigen.



CD4/CD8 coexpression
In the right-hand dot-plot you can see cells that express both the CD4 and the CD8-antigen (arrow) which is highly irregular. In addition both antigens are expressed weakly (compared to normal T-cells). Left-hand panel shows a normal situation.



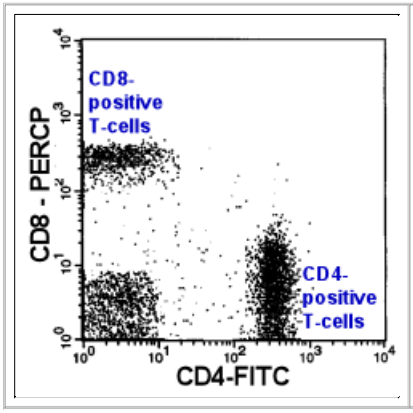
Loss of CD3 Overexpression of CD5
In the right-hand dot-plot you can see T-cells which overexpress CD5 while thy lack CD3 (arrow). Only a few normal T-cells are present. (blue oval). Left-hand panel shows a normal situation.

Caution: As mentioned for B-cells: an abnormal antigen is generally not a reliable sign for T-cell malignancy. Especially if it only concerns a few cells. Unusual T-cell populations may appear in many reactive conditions. In B-NHL we can clear up a suspicious population by defining clonality. In T-cells this usually is not possible. Therefore, in many cases we can only suspect a T-cell lymphoma and suggest further lab tests.

2. Search for monoclonal T-cells

TOP

Monoclonal T-cells are much more difficult to spot than monoclonal B-cells. T-cells have nothing that compares to the kappa/lambda-ratio among B-cells.
The CD4/CD8-Ratio of T-cells (that is the ratio of CD4-expressing to CD8-expressing T-cells) shows some similarity to the kappa/lambda ratio, however, there are certain differences so that the CD4/CD8-ratio is by no way an equivalent tool to detect monoclonality in T-cells. For a start, only extreme alterations of the CD4/CD8-Ratio may be regarded as indicator of T-cell monoclonality. And only if this extraordinary high or low ratio is caused by an increase of a T-cell population.



CD4/CD8-Ratio
Normally, the CD4/CD8-T-Cell ratio in peripheral blood is about 2:1. A In a T-lymphocytic leukemia this ratio can shift dramatically. Unfortunately, this ratio may also be altered by many non-malignant diseases. Therefore, only extreme alterations of this ratio can be regarded as a sign for T-lymphocytic malignancy (and only if the ratio was altered by an increase of one T-cell subgroup and not by a decrease of the other).

Compared to the kappa/lambda-ratio the CD4/CD8-ratio has many disadvantages: The CD4/CD8-Ratio can show strong variations also in non-malignant disease, which means that only extreme alteration may be regarded as indicator of T-cell monoclonality. But even this is only true if the abnormal ratio is caused by an

increase of one of the subpopulations. If it is due to a decrease this is no indicator of monoclonality.
Another drawback is the following: If the kappa/lambda-ratio is equivocal (say a ratio of 5, which may be due to a B-cell lymphoma or it may be not) one often succeeds in isolating the potentially monoclonal B-cell population with some other markers and determine the kappa/lambda-ratio of just this B-cell subpopulation. Then the kappa/lambda-ratio may be 50, and monoclonality of the population has been proven. With T-cells that is different. Of course you can isolate certain T-cell subpopulations and check their CD4/CD8-ratios. But this will not help as many absolutely normal T-cell subgroups consist only of CD4-positive T-cells others only of CD8-positive T-cells. An extreme CD4/CD8-ratio among such a subpopulation is normal rather than a sign of monoclonality.

3. Defining the antigen expression (immunophenotype) of the abnormal T/NK-cells

Having defined the abnormal T-cells one can easily define the other antigens expressed as described for B-cells.

4. Diagnosis of T-/NK-NHL-entity

Preliminary remark
This is a critical point. Can flow cytometry actually establish the diagnosis of a certain T-NHL entity? Not always, strictly speaking not very often. However, flow cytometry results usually make certain lymphoma entities extremely likely and others very unlikely. Therefore, flow cytometry is an important, integral part of lymphoma diagnosis even in cases where it cannot give a definitive diagnosis.

Procedure
In the last chapter we have defined the immunophenotype of the abnormal T-cell population. Now we have to find out which lymphoma entity might show such a phenotype.
To give an idea how that works, a table describing the typical immunophenotypes of the most important mature T-cell neoplasms is shown below.

	T-PLL	T-LGL	NK-LGL	Sezary-Syndrome
CD2	+	+	+	+
CD3	+	**+**	**-**	+
CD4	+/-	-	-	+
CD5	+	-	+/-	+
CD7	**+**	+/-	+/-	**-**
CD8	-/+	+	+/-	-
CD16	-	-/+	+/-	-
CD25	+/-	-	-	-
CD56	-	-	+/-	-
CD57	-	+/-	-/+	-

HLA-DR	-/+	-	-	-
CD4+CD8+ coexpression	25% of cases			
T-PLL: T-Prolymphocytic Leukemia **T-LGL: Large Granular Lymphocyte Leukemia (LGL), T-cell type** **NK-LGL: LGL Leukemia, NK-cell type**				

Seems simple but there are the same problems as described above for B-cells:

- Certain phenotypes are typical but by no means obligatory.
  For example: a Sezary syndrome is usually CD7 negative, but it may be positive. The same is true for practically all other antigens.
- The significance of one marker depends on the presence of other markers
- Not only the presence or absence of an antigen is important but also the strength of its expression (i.e., the antigen density on the cells).

Mentioning these problems shall make clear that interpretation of flow cytometric findings in hematological malignancy is more difficult than suggested by tables like the one above or flow charts that can sometimes be found to guide flow cytometric diagnosis.

V. Flow cytometric analysis of acute leukemia

What are acute luekemias?
Acute leukemias ("acute cancer of the blood") are caused by uncontrolled proliferation of immature blood cells. The designation "acute" stems from the fact that in contrast to chronic leukemia, acute leukemias would lead to death very quickly without therapy.

How are cute leukemias categorized?
The most important categories are the acute myeloid leukemias (AMLs), which are caused by immature myeloid cells, and the acute lymphocytic leukemias (ALLs), caused by lymphatic cells. Further subgroups of these two entities can be defined.

Value of flow cytometric analysis
First, flow cytometry can help detecting an acute leukemia. However, usually this can be done with microscopy as well and in some cases even better. Of greater value is flow cytometry as a fast and reliable method to distinguish between AML and ALL, which is an important decision. Moreover, flow cytometry can help defining the subtype to the acute leukemia, primarily in cases of ALL, to a lesser extent also in cases of AML.

"Blasts"
The immature blood cells of an acute leukemia are generally called blasts. One has to be aware, that in bone marrow a small number of blasts is absolutely normal.

Chapters:

- Steps towards diagnosis
- Finding the blasts
- Defining the antigen expression (immunophenotype)
- Diagnosis

Steps in the flow cytometric analysis of acute leukemia

- **Finding the blast population**
  Are there blasts at all (in the peripheral blood) or are the blasts increased (in the bone marrow)?
- **Defining the immunophenotype** of the blast population
  Which antigens do the blasts express (on their surface and in their cytoplasm)?
- **Diagnosis**
  In virtually all cases flow cytometry can give the answer whether it is an AML or an ALL. In some cases the subgroup can be defined, too.

Note: these steps are not necessarily analytical steps, they are rather a logical concept to approach a diagnosis.

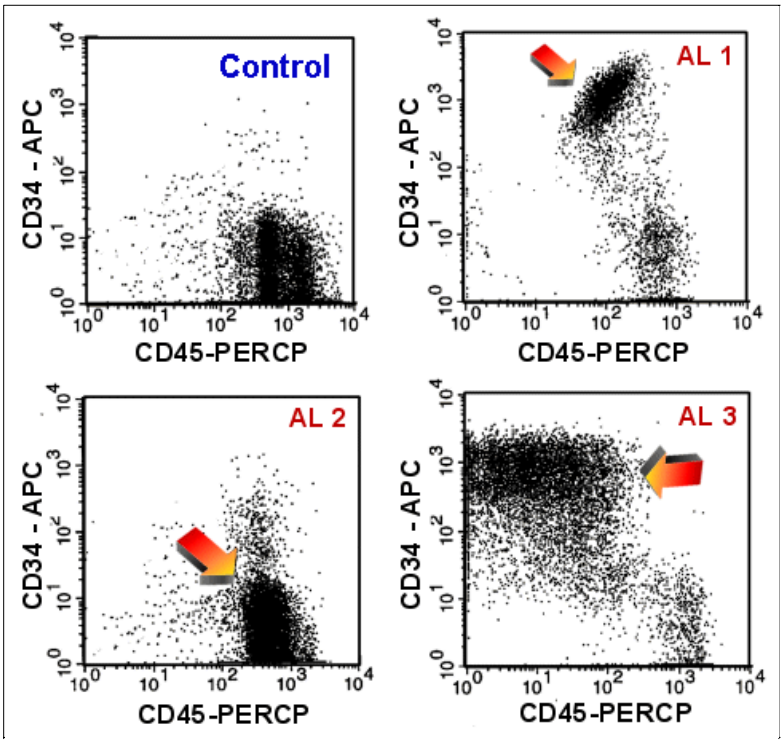
1. Finding the blast population

A malignant blast population may be detected because of

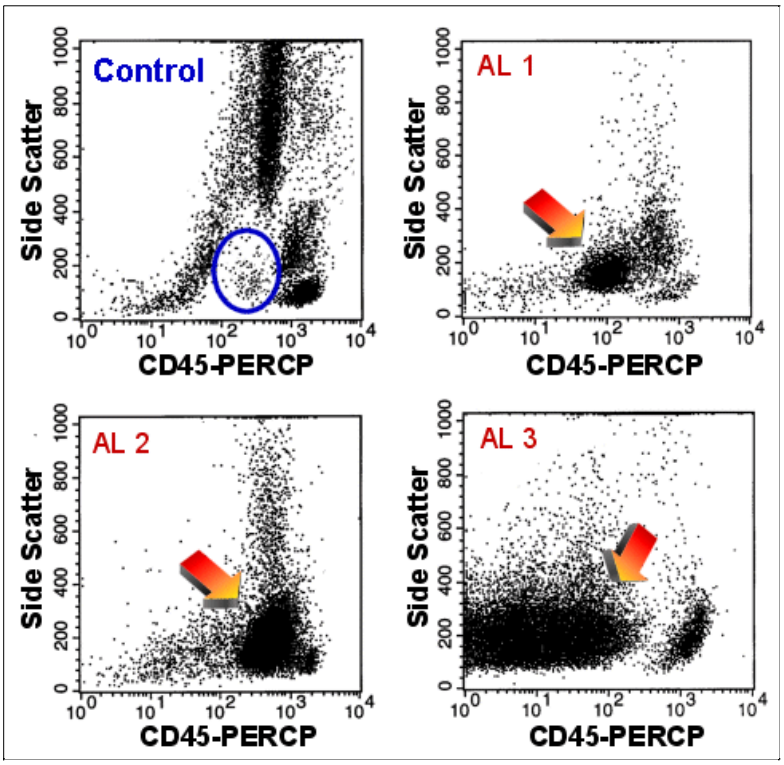
- Increase of immature cells
- Abnormal marker expression of immature cells

a. Conspicuous increase of immature cells
There are many ways to detect immature cells by flow cytometry. One way is to measure expression of antigens that are only present on immature cells, like CD34. Unfortunately this antigen is not expressed on the immature cells in a considerable proportion acute leukemia cases. A more reliable way to look for blasts is to inspect a dot-plot showing CD45-Expression vs. Side Scatter. Immature cells are usually CD45 weaker than lymphocytes and have a low side scatter signal. Therefore, they usually appear in certain spots in the CD45/SSC-dot-plot. With this method one can detect blasts whether they are CD34positive or not. In general one uses both, CD34 expression and the CD45/SSC-dot plot.

Finding immature cells



Finding immature cells using CD45-CD34 dot-plots
A healthy volunteer (upper left) and three cases of acute leukemia. The arrows points at the blast populations, which is very conspicuous in case AL 1 (upper right) and AL 3 (lower right).
In case AL 2 (lower left), the difference between between the normal picture is more subtle and the blasts may be missed because in this case the blasts are CD34 negative.



Finding immature cells using CD45-Side Scatter dot-plots
The healthy volunteer (upper left): he shows only very few cells in the region surrounded by the blue oval.
The three cases of acute leukemia: The arrows point to the blast populations which are clearly visible in all three cases.
Even the blasts of case AL 2 can easily be spotted.

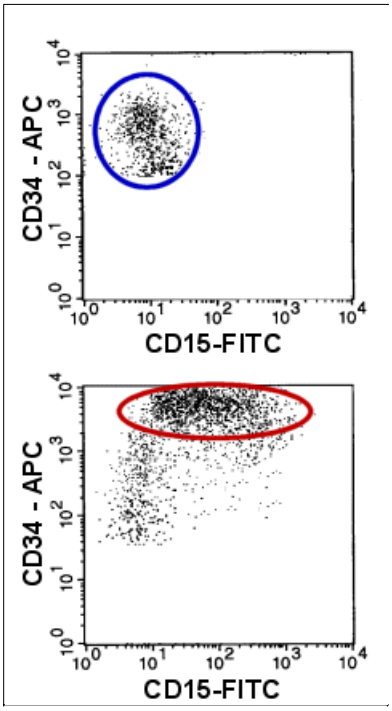
Under normal conditions one finds only very few immature cells in peripheral blood (less than 0.1 % of leukocytes) and even in bone marrow the proportion of immature cells measured by flow cytometry remains relatively low (usually less than 1 or 2 % of leukocytes). An acute leukemia is defined as showing more than 20 % blasts.
At this point it should be mentioned that if a cell type has a high bone marrow / peripheral blood ratio (like blasts or plasma cells) one usually underestimates its proportion in the bone marrow because the bone marrow sample used for flow cytometry is diluted with peripheral blood. Therefore, it may well happen that we find only 6 % blasts by flow cytometry while the pathologist examining the bone marrow biopsy finds 25 %. Simply put: with flow cytometry we examine a bone marrow/blood mixture, the pathologists examine "pure" bone marrow.

b. Abnormal antigen expression of immature cells
There are different kinds of abnormal antigen expression.
The most important are:

- **Expressing antigens of another cell lineage**
  It is abnormal to find lymphatic markers (like CD19, CD7) or markers like CD56 on myeloid cells.
  Inversely, it is also abnormal to find myeloid antigens like CD13 or CD33 on lymphatic cells.

- **Asynchronous expression of antigens**
  CD15 is usually only expressed on more mature neutrophils, finding it on CD34pos blasts is abnormal.

- **Abnormal intensity of antigen expression**
  In some cases of acute leukemia CD34 is expressed much more strongly than on normal blast cells.
  T-ALLs often show a decreased expression of CD3, B-ALL a decreased or lacking expression of CD20.
  Most acute leukemias show a weak some a nearly lacking expression of CD45.



Example of an abnormal antigen expression on myeloid blasts
Compare the normal blasts (upper dot-plot, blue oval) with those of an acute myeloid leukemia (lower dot-plot, red oval)): the malignant blasts abnormally express CD15 and they show an increased expression of CD34.

Note: CD34-negative cells have been removed for reason of clarity.

2. Defining the immunophenotype of the blast population

TOP

Once the malignant blast population is discovered it is usually not very difficult to define its phenotype as described for B-NHLs.

3. Diagnosis

TOP

a. Acute leukemia yes/no?
It may be possible to answer this question with flow cytometry, however, in many cases it may be not. If we find more than 20% blast cells, the criteria of acute leukemia are met. However, if we find less than 20% blast cells it may still be leukemia, as the number of blasts in bone marrow may be considerably underestimated by flow cytometry as explained above. Very mature blast populations may pose another problem.

b. Myeloid or lymphoid blasts?
Flow cytometry can answer this question quickly and reliably. Some antigens are typical for AML others for ALL. Some antigens are typical for B-ALL others for T-ALL.
How reliably a marker defines a lineage can be estimated from a table published by the European group for the immunophenotyping of leukemias (EGIL).
This table was originally published to resolve the problem of acute leukemias carrying the antigens of more than one lineage (biphenotypic leukemias).

EGIL-Score for biphenotypic leukemias

Score	B-lymphocytic	T-lymphocytic	Myeloid
2	CD79 (cyt/membrane) CD22 (cyt/membrane) cyt.IgM	CD3 (cyt/membrane) TCR-a/b TCR-g/d	Myelo-peroxidase (cytopl.)

1	CD19 CD10 CD20	CD2 CD5 CD8 CD10	CD13 CD33 CDw65
0.5	TdT CD24		CD14 CD15 CD64 CD117*

The higher the score for an antigen the higher its value for the lineage assignment. For example, myeloperoxidase (MPO) has the highest score for the definition of AML because it is never present in ALL, CD79 is very important for the diagnosis of B-ALL because it virtually never is expressed in AML or T-ALL.
*Of note, since the original publication of this table CD117 has turned out to be more valuable for detecting myeloid lineage than initially thought. At least one score point for myeloid should be allocated if CD117 is expressed on the blast cells.

c. Definition of the acute leukemia subgroup

AML and ALL are divided in certain subgroups.

- Flow cytometry can elegantly define the subgroups of B-ALL (Pro-B-ALL, common B-ALL, pre-B-ALL and B-ALL) because these groups have been defined according to antigen expression.
- Flow cytometric categorization of T-ALL is slightly less relevant than that of B-cells.
- With AML, flow cytometry can help to detect special subgroups. It is, for example, rather difficult to prove an AML-M7 (megakaryoblastic leukemia) without flow cytometry. Flow cytometry can detect certain antigens (CD41, CD61) on the blast cells which are typical for AML-M7.
  BTW, this sounds easier than it is. This is a very tricky task. One is easily fooled by platelets stuck to the blast cells.

Flow cytometry can also help diagnose other AML-subgroups, but the emphasis is on the word 'help'. These groups are defined by their morphology, cytochemistry or cytogenetics and can, therefore, not be reliably defined by flow cytometry.

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