THESIS

Johanna Frank

2022

Department of Clinical Pathology and Oncology
University of Veterinary Medicine Budapest

Flow cytometry in veterinary medicine – literature review

Written by: Johanna Frank

Supervisor: Márton Márialigeti
Department veterinarian

Budapest, Hungary 2022

Abstract

Flow cytometry is an emerging tool in veterinary medicine. Its application is most widespread in lineage differentiation of hematologic malignancies but other analytical possibilities such as functional assessment of platelets and neutrophil cells can also be performed. With the literature rapidly expanding and the availability of the method increasing it appears valuable to summarize some aspects of this robust technique. This thesis attempts to give an overview of flow cytometry application in veterinary medicine with an emphasis on small animal hematopoietic malignancies. In addition to a methodical description, various conditions are compiled and the use of flow cytometry is described.

Absztrakt

Az állatorvosi gyakorlatban egyre elterjedtebben használt eszköz a flow cytometer. Legkiterjedtebben hematológiai betegségek eredetének feltáráshoz használják. További használati módja lehet egyes sejttypusok,mint a vérlemezkék vagy a neutrophil granulocyták funkciójának vizsgálata. A flow cytometriával foglalkozó állatorvosi irodalom jeletősen novekszik és a módszer egyre inkább elréhetővé válik, ezért célszerűnek látszik ismertetni az ezzel kapcsolatos tudományos irodalmat. Jelen munka a kisállatok hematológiai megbetegdésének előtérbe helyezésével vizsgálja a flow cytometria állatoevosi vonatkozásait. Metodikai leeírás mellett számos megbetegés esetén elírásra kerül a flow cytometria használata.

Table of content

1 List of abbreviations

AL	Acute Leukemia
CBC	Complete Blood Count
CD	Cluster of Differentiation
CETL	Cutaneous Epitheliotropic T-cell Lymphoma
DCS	Dendritic cells
DLBCL	Diffuse large b-cell lymphoma
FACS	Fluorescence Activated Cell Sorting
FC	Flow Cytometry
FCA	Flow cytometric analysis
FIV	Feline immunodeficiency virus
FITC	Fluorescein Isothiocyanate
FNA	Fine Needle Aspiration
FSI	Forward Scatter Intensity
IL	Interleukin
IMHA	Immune-mediated hemolytic anemia
ITP	Immune-mediated thrombocytopenia
LL	Lymphoblastic Lymphoma
MAb	Monoclonal Antibody
MPO	Myeloperoxidase
NK	Natural Killer
PE	Phycoerythrin
RPMI	Rosswell Park Memorial Institute media
r-PLT	Immature reticulated platelets
SSI	Side Scatter Intensity
TCL	T-cell lymphoma
TZL	T-zone lymphoma

2 Introduction

About 50 years ago, Wolfgang Göhde developed the first fluorescence-based flow cytometer in Germany. [1]

Nowadays it is an essential component in research. It has a broad range of applications, especially in the fields of hematology, immunology, and oncology. [2]

„Flow cytometry (FC) is defined as a method for the qualitative and quantitative measurement of biological and physical properties of cells and other particles suspended within a high-velocity fluid stream and passing through a laser beam in a single file. “ [3]

With exceptional sensitivity and specificity, flow cytometry offers the ability to simultaneously assess many properties of individual cells in complicated cell mixtures. This improves the precision of the diagnosis of hematological and lymphoproliferative diseases by making it easier to identify aberrant cells in peripheral blood samples or cell suspensions made from solid tissue biopsies. Researchers and clinicians are interested in using flow cytometry in the diagnosis and investigation of disease due to the growing specialization of veterinary practice. [4]

In the past 30 years, trained professionals were needed to handle the complex and expensive flow cytometers. But with technological improvements, flow cytometry is becoming easier to use and more and more veterinarians are including the technique in their practices. [2] In veterinary oncology, flow cytometry is frequently used to identify and classify hematologic malignancies, especially lymphoid neoplasia including leukemia. It can also be used to evaluate both neoplastic and non-neoplastic marrow diseases. [5]

In this literature review, the application of flow cytometry in veterinary medicine will be summarized. Also, a short explanation of the technology and methods will be included.

3 Flow cytometry

“Flow cytometry is a highly sensitive and specific method for simultaneous analysis of multiple parameters of individual cells in a suspension.” [6]

It is a rapid laser-based technique interrogating several hundred thousand cells individually in a few seconds. [7, 8] It gives an accurate evaluation of the size and the internal complexity of the cells and detects an antigenic pattern that is expressed by each cell and is unique for cellular lineage and the maturation stage. [9]

This technology describes and counts cells with greater accuracy and precision than conventional approaches. [4]

Although flow cytometry is frequently used in research in veterinary medicine, clinical applications and relevance for the characterization of hematolymphoid neoplasms have been developed and increased. [7] In the practice, it is utilized more frequently to confirm diagnoses and characterize the immunophenotype of hematopoietic neoplasms. [9]

The morphological and functional characteristics of cells in the blood, fluid, and tissues of veterinary patients can be determined using flow cytometry. [8]

In small animal medicine, the most frequently used application includes the quantitation of erythrocytes and leukocytes in automated hematology analyzers, the detection of antibodies to erythrocytes and platelets in immune-mediated diseases, the immunophenotyping of leukocytes in hematologic malignancies. [10]

3.1 Components and principles

The fundamental workings of a flow cytometer are as follows: Light from one or more lasers of a certain wavelength interrogates cells or particles in a single file in a fluid media, and light scatter and emission data are recorded. [11]

The main components of a flow cytometer are fluidics, optics, electronics, and a computer to analyze the measured particles/cells. (Figure 1) [12]

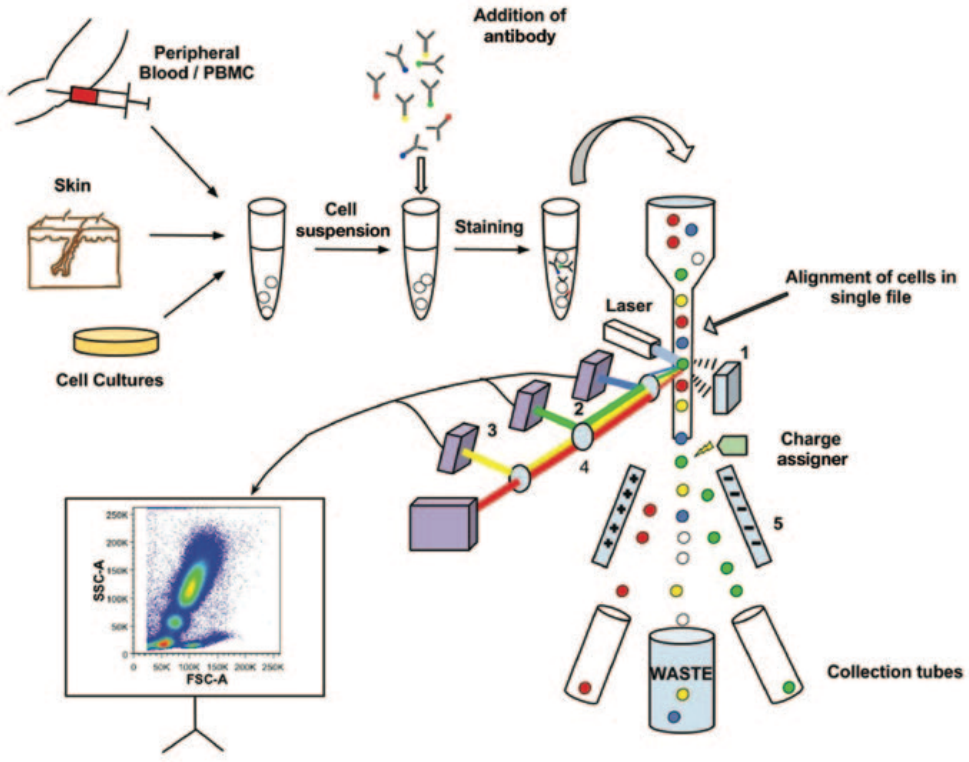


Figure 1: Schematic of flow cytometry system and critical components [13]

3.1.1 The fluidics

In fluidics, samples are suspended in a physiologic fluid and placed into the flow chamber. [11] The flow chamber contains a sheath fluid, a buffered saline solution, which is mixed with the cell suspension. [12] A laminar flow is produced by the moving fluid stream and the cells are lined with even space in the center of the flow chamber so they can be stuck singularly by the laser beam. [11] By setting the flow speed one can adjust the time required for analysis. At the same time, it changes signal characteristics when the particles are investigated individually, and therefore flow speed should be carefully selected.

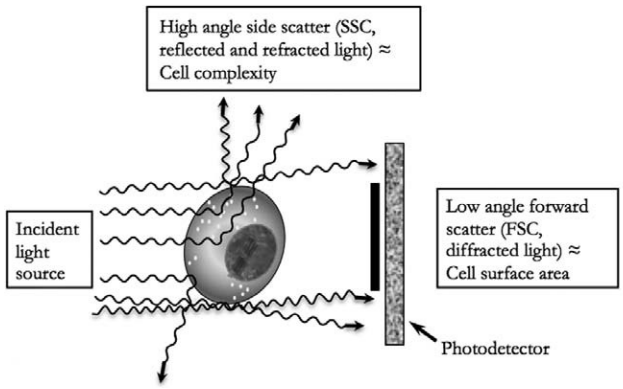
3.1.2. The optics

The optics of the FC include one or more lasers as a light source, and to focus the beam, a set of lenses. [11]

Various lasers with different wavelengths can be used. If a sample is labeled with a fluorescent dye, it will emit light at a specific wavelength. [11] Most fluorochromes, like PE and FITC, absorb the light at 488nm. For ultraviolet-sensitive and far-red fluorochromes, other lasers are required, UV (300-400nm), and far-red (630nm). [12][11]

2 different light scatters can be detected, collected, and analyzed providing information about every single cell. [8] The forward scatter angle is correlated to the size of the cell where the light passes forwards through the cell. The side scatter angle is where the light scatters at a 90-degree angle and it represents the complexity of the cell. (Figure 2) [2] This can be used to help us distinguish for example between granulocytes and lymphocytes. [14] In addition, if the cells are subjected to fluorescently labeled antibodies then the fluorescence of these can be detected too. The detectors apply wavelength filters to detect a narrow frequency range. Various wavelengths of fluorescence for each of the lasers can be used leading to several different signals which may be picked up for analysis. This way, the number of investigated parameters can be increased greatly. An example of fluorochrome application is shown in figure 3. [15] The application of multiple lasers and fluorochromes allows for a multicolor approach. However, the spectrum of emitted light by the fluorochromes is not completely uniform and despite the application of filters, some spillover light may hit other detectors too. Therefore, fluorochrome selection should be cleverly designed and spectral overlap filtered before the analysis of a sample. Fluorochrome selection is facilitated by spectrum viewer tools (for example the BD spectrum viewer, figure 3).

The detected signal strength is dependent not only on the quality of the laser but the number of available molecules for labeling as well as transit time in front of the beam. [12]



2

Figure 2: High and low angle scatter [11]

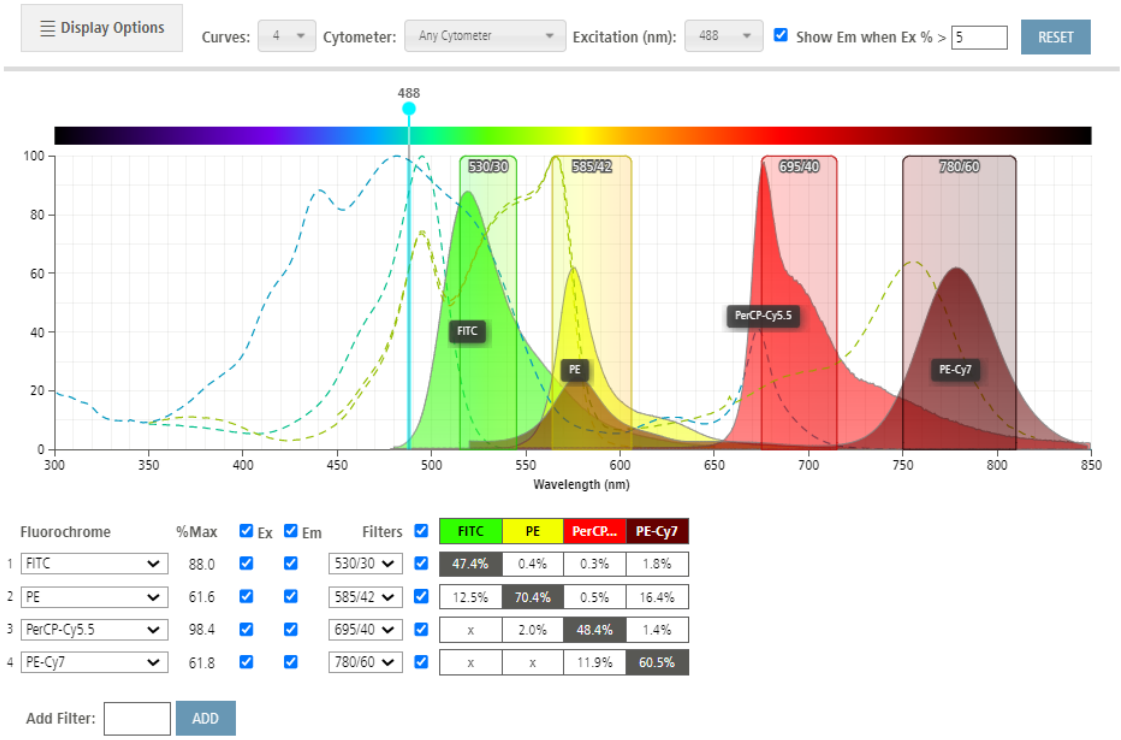


Figure 3: BD Spectrum viewer [16]

Laser	UV Laser (355 nm)		Violet Laser (405 nm)						Blue Laser (488 nm)	
Filter	379/28	450/50	450/50	525/50 505 LP	610/20 595 LP	670/30 635 LP	710/50 690 LP	780/60 750 LP	488/10	530/30 505 LP
Fluorochrome			Pacific Blue	BV510		BV650				FITC
Primary antibody			CD8			Pan B cells				CD21
Method			Covalently labeled in lab	Dye for Live/Dead distinction		Purified mAb/ hybridoma supernatant				Species cross reactive mAb, directly conjugated
Secondary antibody						α-mouse IgM				
Method						Labeled secondary MAb				

Laser	Blue Laser (488 nm)	Yellow/ Green Laser (561 nm)					Red Laser (640 nm)		
Filter	695/40 690LP	586/15	610/20 595LP	670/30 635LP	710/50 690LP	780/60	670/14	730/45 690LP	780/60 750LP
Fluorochrome	PerCP-Cy5.5	PE	Alexa Fluor 594			PE-Cy7	AF647	AF700	
Primary antibody	Integrin α4	Integrin β1	γδ TCR			CD62L	CD4	CD11c	
Method	Species cross reactive mAb conjugated to biotin	Species cross reactive mAb directly conjugated	Purified mAb conjugated with mouse IgG$_1$ Zenon			Purified mAb	Directly conjugated	Hybridoma supernatant conjugated with mouse IgG$_1$ Zenon	
Secondary antibody						α-mouse IgG$_1$			
Method	Fluorochrome labeled streptavidin					Labeled secondary mAb			

Figure 4: Laser spectra, filters, and possible fluorochromes in a specific setup [15]

3.2 Fluorescence-activated cell sorting (FACS)

Fluorescence-activated cell sorting is an application of flow cytometry that separates cells or cell particles, like lymphocytes from a heterogenous suspension and collects them in two or more containers. (Figure 5) [17]

During the process, droplets are formed which pass through the laser and are charged by a charging electrode positively or negatively, based on predetermined characteristics which the flow cytometer can detect. These droplets are then deflected by high-voltage charged plates and collected in tubes according to the desired selection criteria. [12] This application is mostly used in research settings, although practical applications exist too: for example, sperm can be sorted according to sex chromosome content which can be used in reproductive medicine to allow for the determination of the offspring (both in vitro fertilization and insemination procedures). [18]

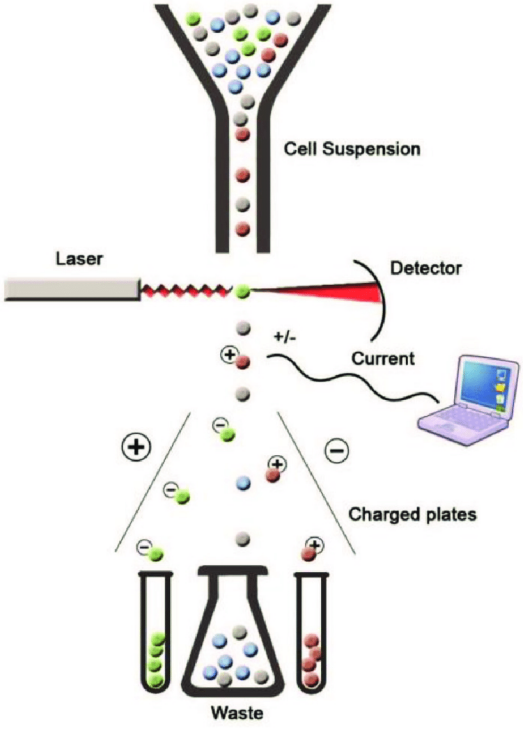


Figure 5: Fluorescent-activated cell sorting (FACS)

The suspended cells are subjected to different fluorescent tags depending on the experimental setup. As the cells flow in a stream of liquid they pass through a laser-detector system that monitors the fluorescent and light scatter characteristics. Based on their characteristics cells are separated in an electric field and into different collecting tubes or multiwell plates. Depending on the experiment a variety of markers may be used for separating the cells of interest. Demonstrated in this figure are cells tagged with green and red fluorescent proteins. Cells that are not labeled are separated into a third column/tube. [19]

3.3 Sample collection

For FC, living cells in a liquid suspension are essential. Samples can be taken from bone marrow, lymph nodes, and blood, but also body fluids and solid tissues. Sufficient cells should be collected without any contamination such as clots or artifacts. [11]

The sample collection, preparation, analysis, and interpretation processes involved in flow cytometry are complicated analytical techniques that have a wide range of potential variables that could have a significant impact on the findings. [7]

Blood samples or bone marrow samples can be collected in EDTA tubes, while lymph nodes or other tissue aspirations are collected like FNA and must be placed in a fluid suspension. A culture medium, fetal bovine plasma, or 1ml saline and 0.1 ml patient serum in a top red tube can be used to transport the FNA. [20]

For an accurate diagnosis, samples from solid tissues should also be prepared and sent for cytology and histopathology.

In the laboratory, RBCs need to be removed from the sample because they are present in a much higher number than leukocytes. Other samples like FNA from solid tissues or cavity fluids can be put into a buffered solution and without or with minimal processing analyzed. [11]

In any case, the viability of the cells must be preserved until analysis. Cells undergo degeneration if they are removed from their natural environment in several ways which may affect the quality of flow cytometric analysis. Markers of interest may be lost or gained, cells (especially neoplastic ones) may disintegrate, and these may lead to inaccurate results (lost data points or debris disturbing the measurement). One may overcome these difficulties by decreasing the sample submission time to the laboratory or with the addition of preservatives to the sample allowing longer transit time. Of these two approaches, the former is more desirable. Preservatives often contain pigments (for instance in the case of the frequently used RPMI 1640 medium) which may increase background fluorescence detected as noise in the detectors. This is especially important in the case of dim signals which may be difficult to separate on analysis. [12] Some leverage may be added by labeling the cells immediately for analysis with which one may only need to remove disintegrated cells from the sample. [21] Even in this case, the storage of the samples in any of these cases should not exceed 48h and samples should be refrigerated. [11]

3.4 Data analysis

The analyzed data can be depicted in histograms or plots. Histograms can only demonstrate one single parameter, in contrast to plots, like dot plots, density plots, or contour plots, which can cross 2 parameters. [17]
A histogram represents one parameter of relative fluorescence or light scatter where multiple samples can be compared. [22]
In a plot, each dot represents a single cell, analyzed by the flow cytometer. [22]

3.5 Gating, data extraction

Gating is an application of flow cytometry that can be used to isolate a subpopulation of cells for analysis. [14]
With this method, unwanted particles such as debris or dead cells can be removed and only the cells of interest remain. [23] The latter especially is of importance, so much so that dead cells are labeled separately.
The data obtained from the analysis consists of forward scatter, side scatter, and often multiple fluorescent signals. With gating one may select a cell population that can be further subdivided into subpopulations of interest. For example, large CD45 (a pan leukocyte marker, see later) positive cells may be selected from a sample and these cells may be subdivided into groups expressing CD3 or CD21, B, or T cell markers. The proportions of these cells can then be quantified. If multiple parameters are analyzed simultaneously then gating strategies may be formulated and the software of the analyzer may store these for use later. Cells are frequently gated based on their size or their expression of the desired marker (for instance CD45 is a pan-leukocyte marker that can be used to select leukocytes from other cells). Gated cells then can be further investigated for the proportionate expression of other characteristic molecules.
In Figure 6 we can see the difference between gating by FSC vs. SSC on the left, where the cell populations cannot be distinguished from each other, and on the right, gating by CD45 vs. SSC for the same event, which is, in this case, the appropriate gating approach. [24]
Furthermore, signals generated by the flow cytometer can be stored in a predetermined file format (*.fcs). These files can be reassessed by other investigators using designated software even without a flow cytometer present. This facilitates proficiency testing and overcomes the

degradability of the samples to a certain extent providing a way to reanalyze the result (including gating). [7]

Automation of gating has been attempted in human medicine with the use of machine learning in some cases. [25] Such an application in veterinary medicine does not appear to exist.

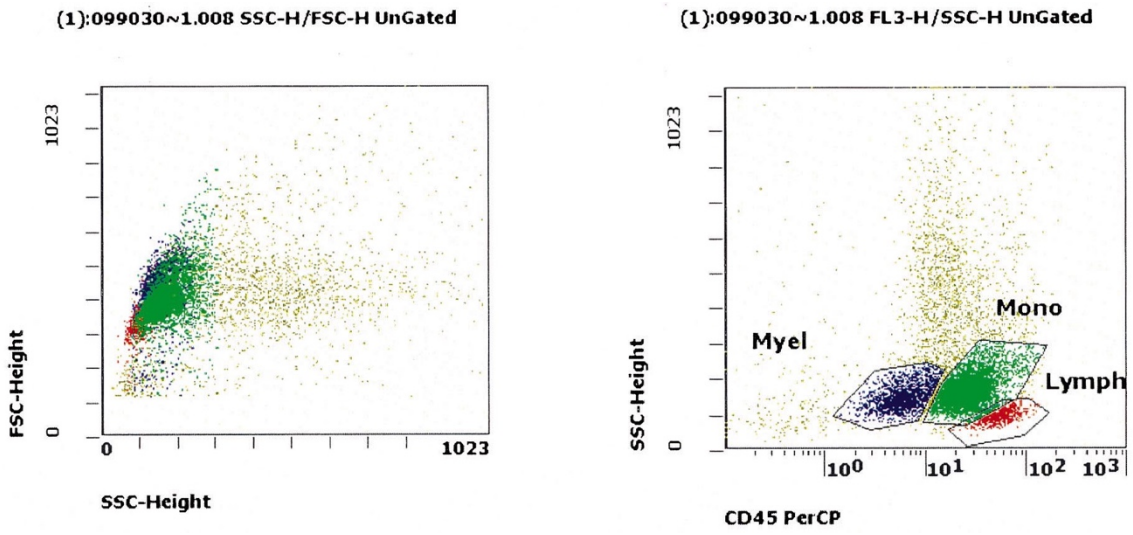
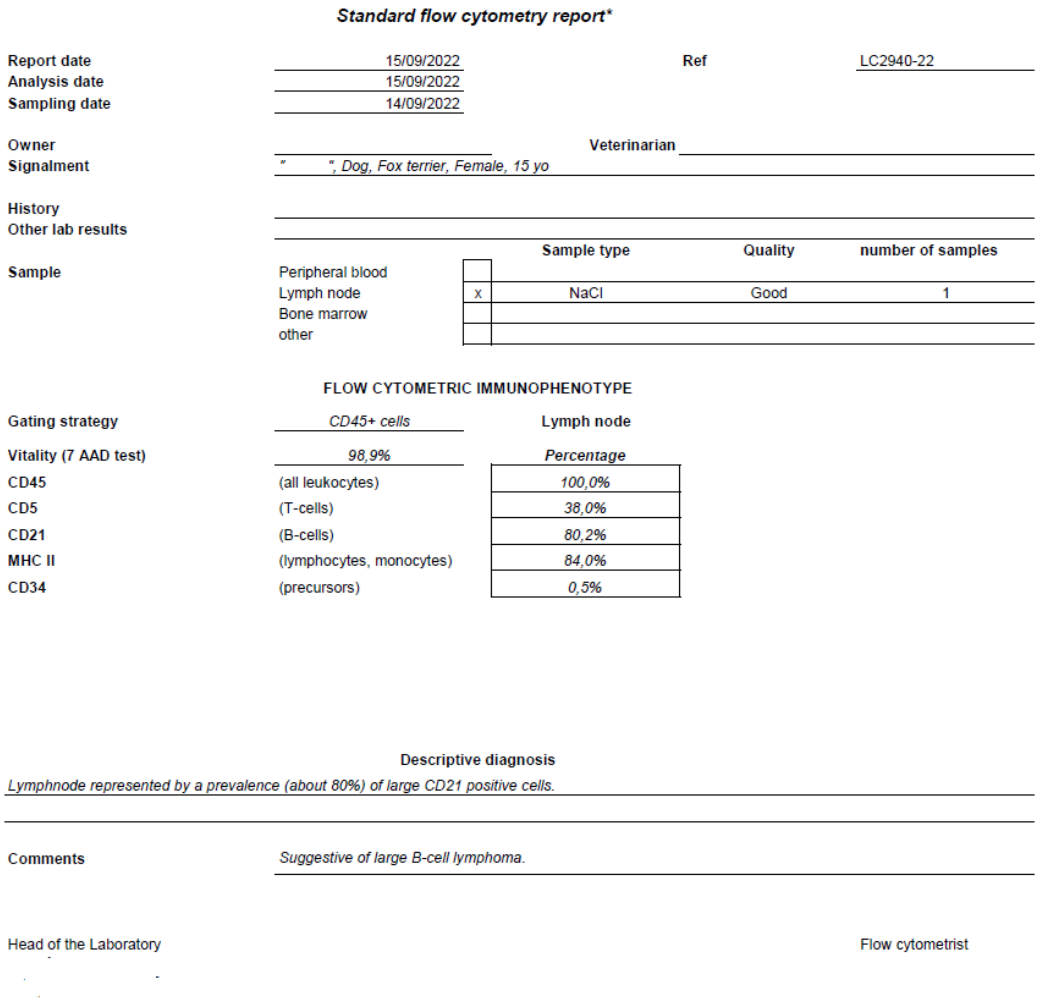


Figure 6: Comparison of gating strategies: FSC vs. SSC on left; CD45 vs. SSC on right. [24]

3.6 Reporting the results

Flow cytometry generates a lot of data. The analysis of such a dataset is performed by trained personnel. However, for clinical applications, the reported results should be comprehensive, yet understandable by the clinician. Members of the European Canine Lymphoma Network realized this need and formulated a consensus statement on the reporting of flow cytometry results. An example is shown in figure 7. [26] According to this, the following pieces of information should be included in a flow cytometry report:

- Laboratory identification
- Patient identification
- Type and quality of the sample(s)
- Sample preparation and staining
- Percentages of positive cells
- Descriptive report
- Diagnosis and interpretation
- Comments and references
- Signatures



Standard flow cytometry report*

Report date	15/09/2022		**Ref**	LC2940-22	
Analysis date	15/09/2022				
Sampling date	14/09/2022				
Owner			**Veterinarian**		
Signalment	" ", Dog, Fox terrier, Female, 15 yo				
History					
Other lab results					

Sample		**Sample type**	**Quality**	**number of samples**
Peripheral blood				
Lymph node	x	NaCl	Good	1
Bone marrow				
other				

FLOW CYTOMETRIC IMMUNOPHENOTYPE

Gating strategy	*CD45+ cells*	**Lymph node**
Vitality (7 AAD test)	*98,9%*	*Percentage*
CD45	(all leukocytes)	*100,0%*
CD5	(T-cells)	*38,0%*
CD21	(B-cells)	*80,2%*
MHC II	(lymphocytes, monocytes)	*84,0%*
CD34	(precursors)	*0,5%*

Descriptive diagnosis

Lymphnode represented by a prevalence (about 80%) of large CD21 positive cells.

Comments *Suggestive of large B-cell lymphoma.*

Head of the Laboratory

Flow cytometrist

Figure 7. Flow cytometry report as recommended by the European Canine Lymphoma Network. [26]

4 Application in veterinary medicine

Flow cytometry-based hematology analyzers are the most widely used application of the technology in veterinary medicine and are found in virtually every veterinary laboratory throughout developed countries. These essentially flow cytometers are limited for a narrow purpose, namely, to recognize erythrocytes, leukocytes, and platelets and allow their differentiation and their morphologic qualities to a certain extent.

Immunophenotyping is a more specialized analysis where cells are differentiated based on their antigen display with the help of monoclonal antibodies and fluorochromes. This technique facilitates the recognition of microscopically indistinguishable cells (for example various lymphocyte subsets). Immunophenotyping is used for the diagnosis and prognostication of hematologic malignancies which may influence treatment choice. Research is ongoing to exploit its full potential.

A third application is found for the detection of antibodies bound to the cellular surface such as immune-mediated hemolytic anemia (IMHA). Immune-mediated thrombocytopenia (IMTP) and antibodies attached to neutrophils were also investigated.

Finally, functional assays are in experimental use for platelet or white blood cell function. [12]

4.1 Immunophenotyping

It is the identification of cell lineage using monoclonal antibodies that identify lineage-specific antigens. The markers can be expressed on the cell surface but also in the nucleus and the cytoplasm of the cell. These markers are called cluster of differentiation (CD). [27] In the human literature at least 376 CD marker was described whereas fewer is described for veterinary species. It is important to note that even if a monoclonal antibody exists for a given CD marker that does not automatically mean that it can be used in flow cytometry. Manufacturers continuously increase the available range for veterinary species. [12]

In veterinary practices, immunophenotyping is the most used application of flow cytometry to analyze cells of the hematopoietic system. [2] Cytology and histopathology are needed for the diagnosis of tumors and with the use of immunophenotyping, tumors, such as lymphomas can be classified and staged. [5] An advantage of flow cytometry compared to histopathology is its relative ease of application and fast turnaround time. Flow cytometry results can be generated within a day from sample collection whereas immunohistochemistry investigation may take a

week. A disadvantage of flow cytometry is the lack of architectural information on the sampled tissue which can be identified in histopathology sections and makes flow cytometry most suitable for the analysis of samples obtained from diffusely affected organs where the target cells are evenly distributed. [12]

4.2 Cluster of differentiation

Table 1: Surface markers of leukocytes in dogs and cats detected using flow cytometry [28, 29]

Canine Markers

Marker	**Cell type**	**Technique**	**Comment**
CD1a	T-cell and B-cell lymphocytes subsets, monocytes, DCs	FCA, ICC	
CD1c	Monocytes, DCs	ICC	
CD3	T cell lymphocytes	FCA, ICC	Membrane, cytoplasm staining pattern
CD4	T-cell lymphocytes, monocytes, DCs, neutrophils*	FCA, ICC	T-helper subsets, *neutrophils in dogs
CD5	T-cell lymphocytes	FCA, ICC	
CD8	Cytotoxic T-cell	FCA, ICC	A cytotoxic subset, NK cells
CD14	Monocytes, macrophages	FCA, ICC	
CD18	All leukocytes	FCA, ICC	Greater intensity on granulocytes and monocytes
CD20	B-cell lymphocytes	ICC	Membrane/cytoplasm staining pattern

CD21	B-cell lymphocytes	FCA, ICC	Absent on plasma cells
CD34	Hematopoietic stem cells	ICC	
CD41	Platelets, megakaryocytes	ICC	
CD42b	Megakaryocytes	ICC	
CD45	All leukocytes	FCA, ICC	
CD61	Platelets, megakaryocytes	FCA, ICC	
CD79a and CD79b	B-cell lymphocytes	ICC	Intracellular antigen
CD117	Mast cells	ICC	
MHC II	Monocytes, DCs, T- and B-cell lymphocytes	FCA, ICC	Membrane/cytoplasm staining pattern
IgM, IgA, IgG	B-cell lymphocytes	ICC	
BLA36	B-cell lymphocytes, DCs	ICC	
Pax5	B-cell lymphocytes	ICC	Nuclear staining
MUM1/RF-4	Plasma cells, B-cell lymphocytes	ICC	Nuclear+/- cytoplasmic staining
Lysozyme	Granulocytes, monocytes, macrophages	ICC	Cytoplasm (granular) staining
MPO	Granulocytes, monocytes	ICC	

Feline markers

Marker	**Cell type**	**Technique**	**Comments**
CD1a	T-cell and B-cell lymphocyte	ICC	

	subsets, monocytes, DCs		
CD1c	Monocytes, DCs	ICC	
CD3	T-cell lymphocytes	ICC	Membrane/cytoplasm staining patterns
CD4	T-cell lymphocytes, activated monocytes, macrophages, DCs, neutrophils	FCA, ICC	
CD5	T-cell lymphocytes	FCA, ICC	
CD8	T-cell lymphocytes	FCA, ICC	A cytotoxic subset, NK cells
CD11b	Granulocytes, monocytes, macrophages	FCA, ICC	
CD11d	T-cell lymphocytes subset, monocytes, and histiocytes	ICC	
CD14	Monocytes, macrophages	FCA, ICC	
CD18	All leukocytes	FCA, ICC	Greater intensity on granulocytes and monocytes
CD20	B-cell lymphocytes	ICC	Membrane/cytoplasm staining pattern
CD21	B-cell lymphocytes	FCA, ICC	Absent on plasma cells
CD22	B-cell lymphocytes	FCA	Absent on plasma cells
CD45	All leukocytes	FCA	
CD79a and CD79b	B-cell lymphocytes	ICC	Intracellular antigen

CD103	Intra-epithelial lymphocytes	FCA	
CD117/c-kit	Mast cells	ICC	
BLA36	B-cell lymphocytes, dendritic cells	ICC	
Pax5	B-cell lymphocytes	ICC	Nuclear staining
MUM1	Plasma cells, B-cell lymphocytes	ICC	Nuclear +/- cytoplasmic staining pattern
MHCII	Monocytes, DCs, T- and B-cell lymphocytes	FCA, ICC	Membrane/cytoplasm staining pattern
Lysozyme	Granulocytes, monocytes, macrophages	ICC	Cytoplasmic (granular) staining
MPO	Granulocytes, monocytes	ICC	
Tryptase	Mast cells	ICC	

In the tables above, the most used cluster of differentiation in veterinary medicine is listed.

4.3 Lymphoma

In dogs and cats, the most frequent hematologic malignancies are lymphomas, and malignant proliferations of lymphoid cells, arising from solid tissues. [30–32] Lymphomas are a diverse group of malignancies bearing the common characteristics of the expansion of neoplastic lymphoid cells either in tissues where lymphoid cells are expected (i.e., lymph nodes) or where they are unexpected (i.e., liver, kidneys, etc.) in large numbers. Considering that a lymphoid malignancy arises from a single clone (clonality) it is straightforward that neoplastic lymphoid cells possess the same immunophenotype which can be used for the diagnosis of the disease. Although demonstrating clonality by flow cytometry is difficult (see later) the expansion of cells with similar morphology and immunophenotype is often sufficient to diagnose the disease. [12]

To classify lymphomas, FNA from enlarged lymph nodes or masses are taken, placed into a suitable container with buffer saline, stained with fluorescent antibodies, and analyzed by flow cytometry. [11] It is used to emphasize the results from histopathology or cytology, and to examine the immunophenotype of the cells. [33] In correlation to other techniques, FC is simple and quick to use, and with the application of gating, a mixed cell population can be analyzed. [34]

Flow cytometry can be used for lineage assessment: knowledge on whether the lymphoma is T-cell, B-cell, or NK cell origin carries information regarding optimal treatment modality and prognosis of survival and response to therapy. [34] [20] Markers expressed on one cell line and absent on the other can be used for lineage assessment. B cells express CD20, CD21, CD79a, IgG or IgM whereas T-cells express CD3, CD4, CD5 or CD8.

This property can be enhanced greatly if the actual subtype is known, which can be achieved by flow cytometry in certain cases, especially if flow cytometry is coupled with morphologic analysis of the cells (cytology). For example, the survival of patients suffering from T-cell lymphoma is generally shorter than that of the B-cell counterpart; however, small clear cell lymphoma, a T-cell variant, carries a much better prognosis. [27]

Cellular maturation can be assessed too which may help differentiate leukemia from stage V lymphoma (advanced lymphoma manifesting in the bone marrow and often in the bloodstream). It is known that lymphoid cells are losing CD34 positivity upon leaving the bone marrow. Lymphomas arising from peripheral organs are therefore CD34 negative whereas leukemias arising from the bone marrow are CD 34 positive. [35]

In select cases, aberrant immunophenotypes of lymphoid cells can be detected which can further increase diagnostic accuracy. An aberrant immunophenotype is characterized by an unexpected expression of a marker. This might either be an under- or overexpression of a typical marker for a lymphoid malignancy or an expression of a marker that normally isn't found in the investigated population. For example, T-zone lymphoma in dogs is characterized by the loss of CD45, the common lymphocyte antigen, and an aberrant expression of CD21 which is unexpected in T-cell lymphoma. [34]

The use of flow cytometry has been attempted to detect minimal residual disease in lymphoid tumors. Minimal residual disease detection is routinely performed in human medicine but is rarely investigated in veterinary settings. Flow cytometry with careful settings can detect as few as 1 neoplastic cell in 10000 cells which improves sensitivity compared to traditional methods like cytology (5 in 100 cells). [27, [36]

The advantages of flow cytometry can mostly be used if a diffuse neoplastic infiltrate is present in the investigated tissue. In dogs, this contributes to the useability of flow cytometry because several lymphoma subtypes are diffusely infiltrating. At the same time, this limits the applicability of flow cytometry in cases where neoplastic cells are similar in size to their healthy counterparts or when the neoplastic cells are present in a low proportion. Such an example can be that of the indolent lymphomas without an aberrant immunophenotype and for the latter the dilemma of a reactive lymph node. Therefore, it appears wise to have information about the composition of healthy lymphoid organs for comparison which enables the detection of a larger proportion of cells with similar immunophenotypes, a hallmark of neoplastic expansion. Such studies were carried out for healthy canine and feline peripheral blood, lymph nodes, and canine bone marrow. [17, [37] [38] Although this might serve as some form of a reference interval similar to laboratory analytes clinical cutoff values are needed to determine what proportion of marker expression may be atypical.

4.3.1 B-cell lymphomas in dogs

There are different types of B-cell lymphomas, such as marginal zone, follicular, mantle cell, and others. The most common subtype in dogs is diffuse large B-cell lymphoma (DLBCL). [39] As a study shows, some breeds are more prone to this malignancy, such as Basset Hound, Border Collie, Cocker Spaniel, Standard Schnauzer, and others. [40]

Markers used for B-cell lymphomas are CD20, CD21, CD79a IgG, and IgM. [34]. An obvious abnormal antigenic pattern is needed for the definite diagnosis of a neoplastic population. If it is absent then other methods, such as cytology, histopathology, and immunohistochemistry are essential for the correct diagnosis. [41] Flow cytometry and cytology are often used as adjunct diagnostic methods and are sufficiently robust to detect several B-cell tumors.

In Figure 8 a forward scatter vs. side scatter was applied from a lymph node aspirate for the evaluation of the cell size. [41]

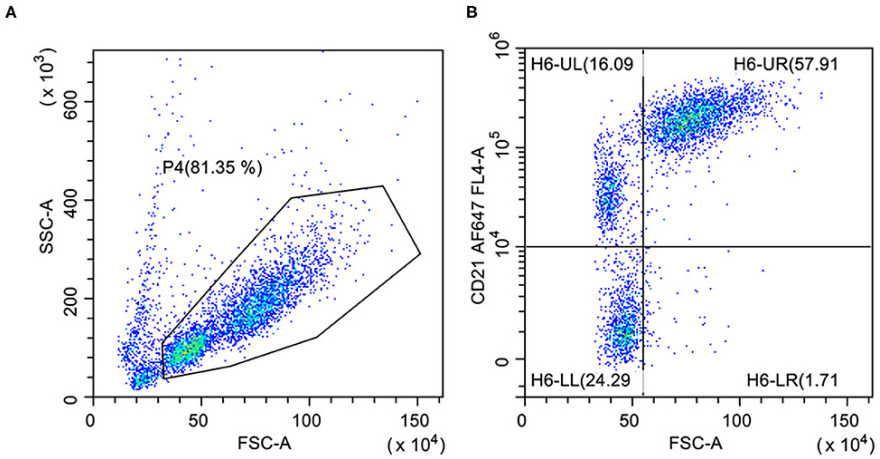


Figure 8: Flow cytometric presentation of a large B-cell lymphoma. (A) Forward scatter (FSC) vs. side scatter (SSC) plot after doublet exclusion showing two populations of small- and large-sized cells. (B) FSC vs. CD21 plot of P4-gated cells showing higher CD21 expression on large cells (H6-UR) compared with small B-lymphocytes (H6-UL). [41]

4.3.2 T-cell lymphomas in dogs

Canine T-cell lymphomas – similarly to B-cell tumors - are heterogenous malignancies. [42] They originate from T-cells and represent about 30-40% of canine lymphomas. [43]

There are different subgroups of T-cell lymphomas. The most common ones are peripheral T-cell lymphoma (PTCL) and T-zone lymphoma (TZL). [43] Others are less frequently encountered but carry a poor prognosis: lymphoblastic lymphoma (LL) and cutaneous epitheliotropic T-cell lymphoma (CETCL) are aggressive tumors. As a study shows, some breeds are more prone to T-cell lymphoma than others like Siberian Husky, Irish Wolfhound, Airedale Terrier, Cavalier King Charles Spaniel, Shih Tzu, and some others. [40]

In Table 1 above the most used antibodies for the characterization of T-cells with FC are listed, such as CD3, CD4, CD5, CD8, MHC II, etc.[43]

With flow cytometry, we can easily distinguish between reactive and neoplastic T-cells by using a multicolor approach. Lymph node hyperplasia can be due to a chronic process such as Leishmania, viral infections, or dermatitis. As a result, lymph node hyperplasia will have a mixed cell population, contrary to PTCL where only the T-cell will be increased. [43]

Flow cytometry, histopathology, and cytomorphology can be used for the classification of TCL subtypes, such as PTCL, LL, TZL, CETL, etc.

PTCL is one of the most common subtypes of TCL with different outcomes. In Figure 9 flow cytometric characteristics of PTCL (CD3+, CD4+, CD45+, and MHCII-) are demonstrated. [43]

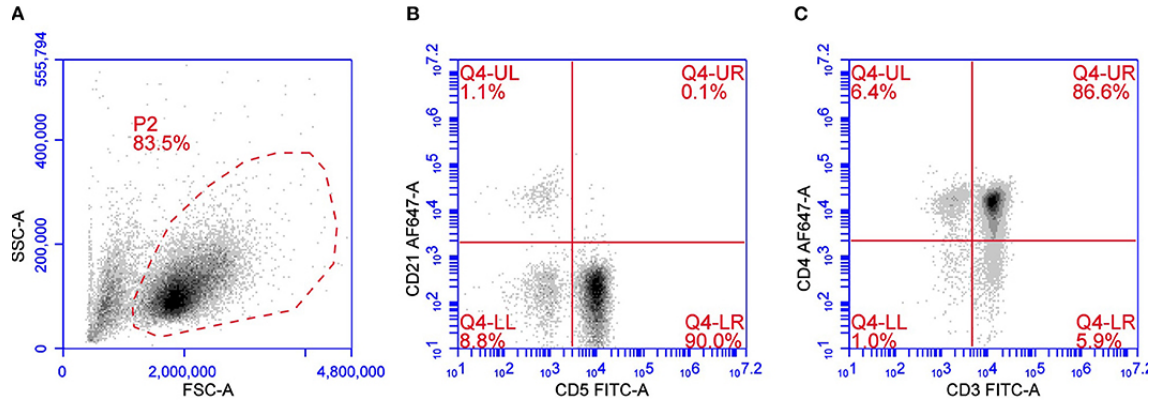


Figure 9: Flow cytometric presentation of a PTCL. (A) Forward scatter (FSC) vs. side scatter (SSC) plot after doublet exclusion showing a unique population of medium-to-large sized cells. (B, C) CD5 vs. CD21 plot (B) and CD3 vs. CD4 plot (C) of P2-gated cells showing a predominant population of CD5+CD3+CD4+ cells. [43]

In only 3% of canine lymphomas, we can see LL. Lymphoblastic lymphomas arise from precursor cells, are very aggressive, and have a poor outcome. FC is used to grade the malignancy. [43]

Also, TZL is rare and small neoplasms. As visible in Figure 10, we can quickly identify TZL with a multicolor approach, the distinctive loss of CD45 expression, and the abnormal expression of CD21, a B-cell marker.

FC is essential to define the immunophenotype of the tumor which is necessary for the proper therapy. We can also differentiate TZL from PTCL and classify the subtypes of TCL [43]

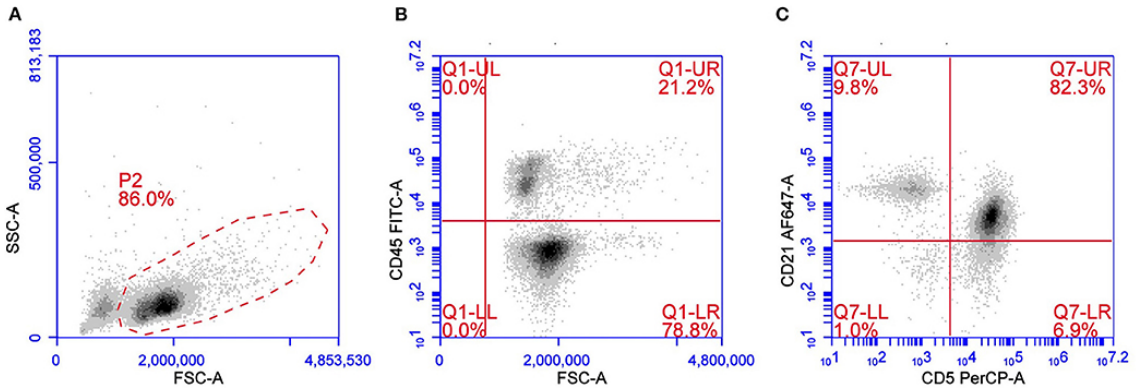


Figure 10: Flow cytometric presentation of a TZL. (A) Forward scatter (FSC) vs. side scatter (SSC) plot after doublet exclusion showing a prevalent population of medium-sized cells. (B, C) FSC vs. CD45 plot (B) and CD5 vs. CD21 plot (C) of P2-gated cells show that medium-sized cells are CD45–CD5+CD21+dim.[43]

4.3.3 Case study: Beagle with B-cell lymphoma and T-cell acute lymphoblastic leukemia

In rare cases, the simultaneous appearance of lymphoid malignancies may result in conflicting results on flow cytometry. Lineage differentiation in these cases may be more difficult. Such a case is described by Ferrari A., et al. [44] A Beagle was presented with lethargy, weight loss, and peripheral lymphadenomegaly. In addition to a complete blood count and biochemistry, cytological examination, US, and fine needle aspiration of lymph nodes were taken to perform FC immunophenotyping. To stage the disorder, samples from the bone marrow and blood were taken and sent for flow cytometric analysis. As a result, a CD21-positive large cell population can be seen in Figure 11 which demonstrates a large B-cell lymphoma. On the other hand, a different lymphoid cell population has been demonstrated from blood and bone marrow for which a different antibody panel was used. These cells were positive for pan leukocyte markers CD45, CD44, and CD18 but negative for myeloid markers and CD21. Weak positivity for T-cell markers was observed (14% positive cells for CD5). Although cutoff values for marker positivity in veterinary literature are lacking, it is generally considered in human medicine that 20% is the threshold for a given marker and is used by some experts in flow cytometry in veterinary medicine. [27] In this case, additional diagnostics were used to determine the final diagnosis (PCR for receptor rearrangement) of the concomitant appearance of B- and T-cell lymphoid malignancies. [44]

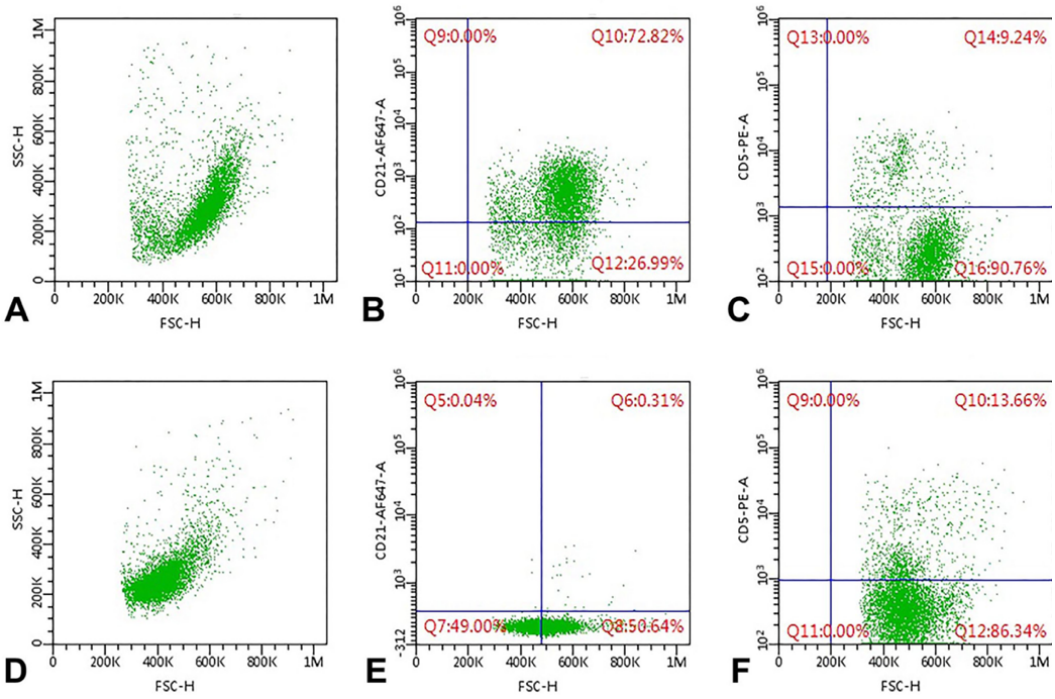


Figure 11: Flow cytometric analysis of a lymph node (LN; A–C) aspirate and a bone marrow (BM; D–F) sample from a dog with concomitant large B-cell lymphoma and acute leukemia of suspected T-cell lineage.

Only CD45-positive cells are shown. Discriminators were set based on negative unstained controls. A. Scattergrams of the LN aspirate; cells are displayed based on cell size (FSC-H) and complexity (SSC-H). B. Most of the cells in the LN aspirate were large and stained positive for CD21 (upper right quadrant). C. A few small cells in the LN aspirate stained positive for CD5 (non-neoplastic residual T cells, upper right quadrant). D. Scattergrams of the BM sample; cells are displayed based on cell size (FSC-H) and complexity (SSC-H). E. Large cells staining positive for CD21 represented <1% of the cell population in the BM sample (upper right quadrant). F. A subset of neoplastic cells in the BM sample stained positive for CD5 (upper right quadrant).[44]

4.3.4 Flow cytometry for lineage determination in lymphoma in cats

In cats, a case study of 19 cats was made for the diagnosis of lymphoproliferative disorders with the use of FC for immunophenotyping.

As a result of clinical and laboratory findings, such as lymphocytosis, peripheral lymphadenopathy, abdominal-, mediastinal- and retrobulbar masses, enlarged kidneys, thickened intestinal wall, and pleural effusion, which were indicating lymphoid neoplasia, referring veterinarians suggested FC. Cats of different breeds, like British-, domestic shorthair, Persian, Bengal, etc., and different age groups, from 6 months up to 16 years, female neutered and male neutered, attended the study.

The cats were divided into 2 groups, in group 1 were cats with formerly diagnosed lymphoma, and in group 2 were cats with non-neoplastic lymphoproliferative disorders.

Not only FC, but also other tests were formerly carried out such as hematology, biochemistry, diagnostic imaging, cytology, and histology. The cats also tested negative for FeLV and FIV.

A sample with FNA aspiration was taken and processed by a laboratory. With the use of monoclonal antibodies, CD4, CD5, CD8, and CD21 flow cytometric measurements were performed. With the light scattering properties of the lymphoid cells and to analyze the expression of surface antigen, they used the gating technique.

In the results of group 1 which we can see in figure 12, in 12 out of 13 lymphoma patients, FC was able to determine the cell lineage. One patient presented with multicentric lymphoma/leukemia, one with extranodal lymphoma, two with mediastinal lymphoma, three with multicentric lymphoma, and six with alimentary lymphoma. [31]

						Phenotype of lymphoma				
No.	**Age**	**Sex**	**Breed**	**Sample analysed**	**Lymphoma subtype**		**CD4**	**CD5**	**CD8α/β**	**CD21**	**Definitive diagnosis based on**
1	7	MN	DSH	Intestinal mass	Alimentary lymphoma	B cell	–	–	–	+ (95%)	Intestinal mass FNAC
2	12	MN	DSH	Intestinal mass	Alimentary lymphoma	B cell	–	–	–	+ (92%)	Intestinal mass FNAC
3	8	FN	DSH	Gastric mass	Alimentary lymphoma	B cell	–	–	–	+ (96%)	Intestinal mass FNAC
4	12	FN	DSH	Intestinal mass	Alimentary lymphoma	B cell	N/d	–	N/d	+ (95%)	Intestinal mass FNAC
5	15	MN	Persian	Intestinal mass	Alimentary lymphoma	B cell	N/d	–	N/d	+ (91%)	Intestinal mass FNAC
6	9	MN	DSH	Mesenteric lymph node	Alimentary lymphoma	T cell	N/d	+ (93%)	N/d	–	Lymph node FNAC
7	16	FN	DSH	Spleen	Multicentric lymphoma/leukaemia	T cell	+ (94%)	+ (95%)	–	–	Spleen and liver FNAC
8	2	MN	DSH	Mesenteric lymph node	Multicentric lymphoma	T cell	–	+ (94%)	+ (93%)	–	Lymph node FNAC
9	10	MN	BSH	Intestinal mass	Multicentric lymphoma	B cell	N/d	–	N/d	+ (90%)	Intestinal mass FNAC
10	0.5	MN	DSH	Peripheral lymph node	Multicentric lymphoma	UTD	+ (31%)	+ (53%)	+ (15%)	+ (32%)	Lymph node histology and IHC (B cell multicentric lymphoma)
11	1	MN	DSH	Pleural fluid	Mediastinal lymphoma	B cell	–	–	–	+ (90%)	PARR of pleural fluid
12	1	FN	DSH	Pleural fluid	Mediastinal lymphoma	T cell	+ (99%)	+ (93%)	–	–	Mediastinal mass FNAC and pleural fluid analysis
13	5	FN	Bengal	Retrobulbar mass	Extranodal lymphoma – retrobulbar	B cell	N/d	–	N/d	+ (92%)	Retrobulbar mass FNAC

Figure 12: Characteristics (age, sex, breed, sample type, lymphoma subtype, phenotype, diagnostic tests used for the definitive diagnosis) of cats with lymphoma (group 1) [31]

								Phenotype of lymphocytes			
No.	**Age**	**Sex**	**Breed**	**Sample analysed**	**Reason for performing immunophenotyping**	**Lymphoproliferative disorder excluded by**	**Disease recognized**		**CD4**	**CD5**	**CD8α/β**	**CD21**
14	13	FN	DSH	Mesenteric lymph node	Generalised lymphadenomegaly	Lymph node FNAC	Reactive lymphoid hyperplasia	Mixed	+ (32%)	+ (51%)	+ (22%)	+ (42%)
15	9	MN	DSH	Peripheral lymph node	Lymphadenomegaly	Lymph node FNAC	Reactive lymphoid hyperplasia	Mixed	+ (33%)	+ (65%)	+ (37%)	+ (28%)
16	1	FN	DLH	Peripheral lymph node	Generalised lymphadenomegaly	Lymph node FNAC	Reactive lymphoid hyperplasia	Mixed	+ (21%)	+ (53%)	+ (26%)	+ (44%)
17	4	MN	DSH	Mesenteric lymph node	Thickening of intestinal wall and mesenteric lymphadenomegaly	Intestine and lymph node FNAC	Reactive lymphoid hyperplasia	Mixed	N/d	+ (29%)	N/d	+ (57%)
18	10	FN	DSH	Spleen	Recurrence of IMHA, suspicion of multicentric lymphoma	Spleen histology	IMHA	Mixed	+ (57%)	+ (85%)	+ (18%)	+ (10%)
19	7	FN	DSH	Pleural fluid	Chylothorax	Pleural and abdominal fluid analysis	Idiopathic chylothorax	Mixed	+ (30%)	+ (55%)	+ (26%)	+ (24%)

Figure 13: Characteristics (age, sex, breed, sample type, reason for performing immunophenotyping, diagnostic tests used for the definitive diagnosis, disease recognized, phenotype) of cats with non-neoplastic lymphoproliferative disorders (group 2) [31]

The result of group 2 in figure 13, the cats presented idiopathic chylothorax, systemic inflammatory response secondary to immune-mediated hemolytic anemia, and reactive lymphoid hyperplasia. [31]

FC immunophenotyping is an effective and adequate approach for the diagnosis of lymphoproliferative disorders as this study shows. In all cases, other diagnostic tests were applied to back the diagnosis.

Cytology of FNA in combination with immunophenotyping of a mass or lymph node can give a quick and accurate diagnosis in comparison to a histological sample, where the patients need sedation/general anesthesia, which is more invasive, takes more time, is pricier, and often not enough for an appropriate diagnosis.

The main advantages of flow cytometry in cats are its high sensitivity, specificity, quick analysis time, and ease of sampling from various tissues like kidneys, bone marrow, peripheral blood, etc. [31]

4.4 Leukemia

Although lymphoma and lymphoid leukemia is often considered to be the same disease and distinct from myeloid neoplasms like acute or chronic myelogenous leukemia, their differentiation is often quite difficult clinically. In addition, stage V lymphoma invading bone marrow and potentially blood may prove to be an additional difficulty. Furthermore, non-neoplastic expansion of lymphoid cells is known to appear. [45] Neoplastic cells of different lineages in bone marrow or the blood may look very similar under the microscope. However, their immunophenotypic characteristics may be quite distinct. Flow cytometry can be an optimal tool for their differentiation.

4.4.1 Chronic leukemia

Lymphoid and myeloid cells in cases of chronic leukemias can be easily distinguished morphologically. The appearance of the neoplastic cells is often very similar to that of their benign counterparts. Continuous increases in one cell type should raise suspicion for chronic leukemia. Inconclusive cases may be investigated with flow cytometry. However, in these

cases, the use of flow cytometry is limited to the determination of lineage. Defining neoplasia itself often requires clonality testing by PCR, as demonstrated in the case of English bulldogs where a non-neoplastic polyclonal lymphocyte expansion was described, potentially with a genetic basis which is easily confused with chronic B-cell lymphoid leukemia both morphologically and flow cytometrically. Microscopically the cells resembled chronic lymphocytic leukemia and flow cytometry demonstrated CD25 positivity and MHCII positivity, none of which is distinctive for neoplasia itself. Therefore, in this case, PCR for receptor rearrangement (PARR) is required for a definite diagnosis. [45]

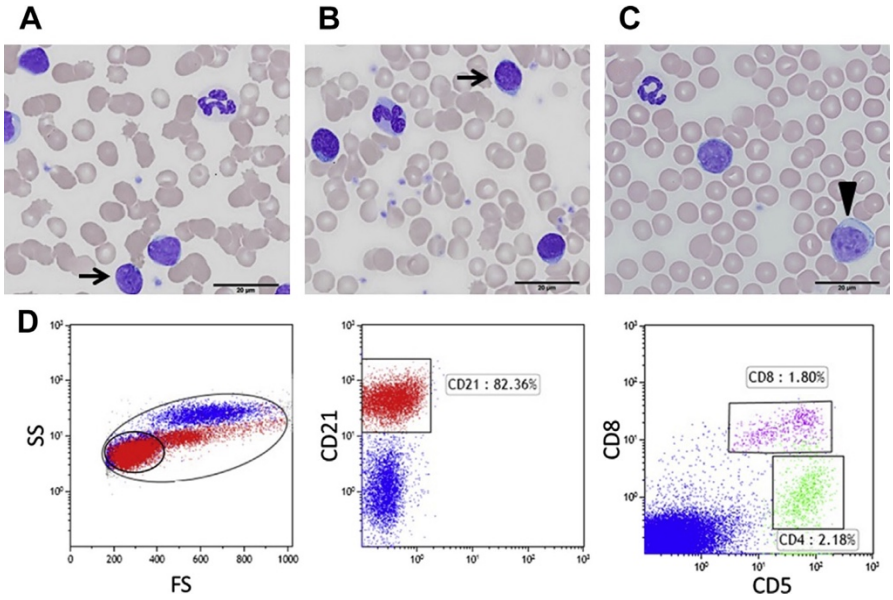


Figure 14: Peripheral blood cytology (A–C) from 3 cases of canine B-cell chronic lymphocytic leukemia and characteristic flow cytometric findings (D). The majority of lymphocytes are small and well-differentiated (arrow) with rare larger more immature cells (arrowhead). The majority of CD21 cells (red) fall within the small size gate, indicated by the small black circle, with small numbers of cells extending into the monocyte region. [46]

B-cell CLL is commonly seen in older, small-breed dogs with different clinical findings such a, peripheral and visceral lymphadenopathy, splenomegaly, and in some cases cytopenia and hyperglobulinemia. Neoplastic small, well-differentiated B-cell inflate the peripheral blood. [46]

As visible in figure 14, in B-cell CLL a large population of mature small lymphocytes can be expected. [46]

T-cell lymphocytic leukemia is more commonly seen in dogs than in B-CLL. Older, large-breed dogs are affected by malignancy with clinical signs such as anemia and splenomegaly. [46]

In Figure 15, T-CLL cells with an intermediate size and a pale blue cytoplasm are visible. The forward and side scatter properties of the cells differ by flow cytometrical analysis. [46]

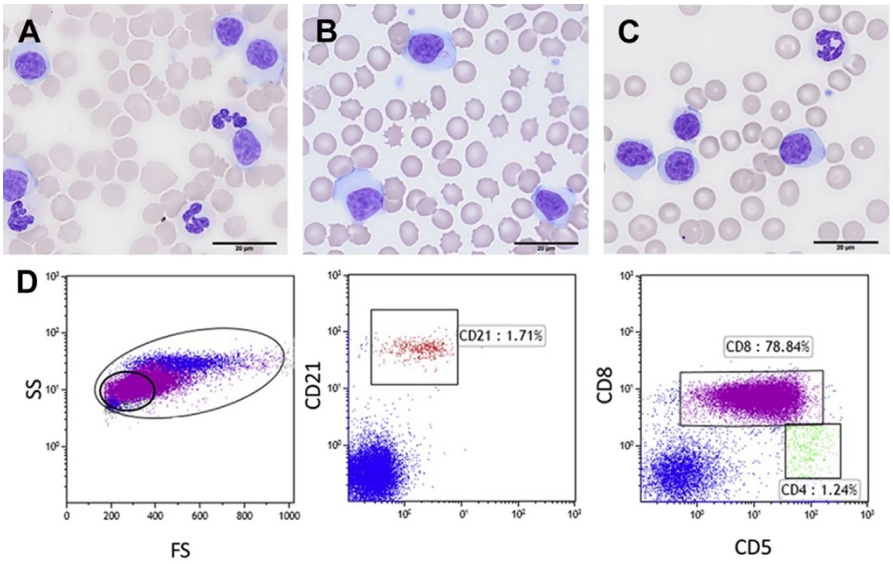


Figure 15: Peripheral blood cytology (A–C) from 3 cases of canine CD8 T-cell leukemia and characteristic flow cytometry findings (D).

The majority of lymphocytes are intermediate in size with mature chromatin and abundant pale cytoplasm. Cases vary from having few granular cells (left) to having predominantly granular cells (right). By flow cytometry, CD8+ CD5 T cells (magenta) have to scatter properties similar to normal small lymphocytes and monocytes. These cells express CD45. [46]

4.4.2 Acute leukemias and stage V lymphoma

Acute leukemia is caused by hematopoietic cell deviation arising from the blood or bone marrow.[32, 47] It is a heterogeneous group of aggressive neoplastic diseases of immature hemopoietic cells divided into 3 subtypes, acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and acute undifferentiated leukemia. [46, 47] Clinically acute leukemias present with cytopenia other than the neoplastic cell line, no evident peripheral lymphadenopathy, and circulating blast cells. [46]

Acute leukemia differentiation on the other hand appears to be more difficult with microscopic analysis alone. It is complicated further with the case of advanced lymphoma which may appear in bone marrow or blood. An expansion of blast cells in these cases is usually straightforward with microscopy, but lineage determination often proves very difficult if not impossible.

ALL and stage V lymphomas are mostly differentiated by peripheral lymph node involvement. However, acute lymphoid leukemia may lead to lymph node enlargement.

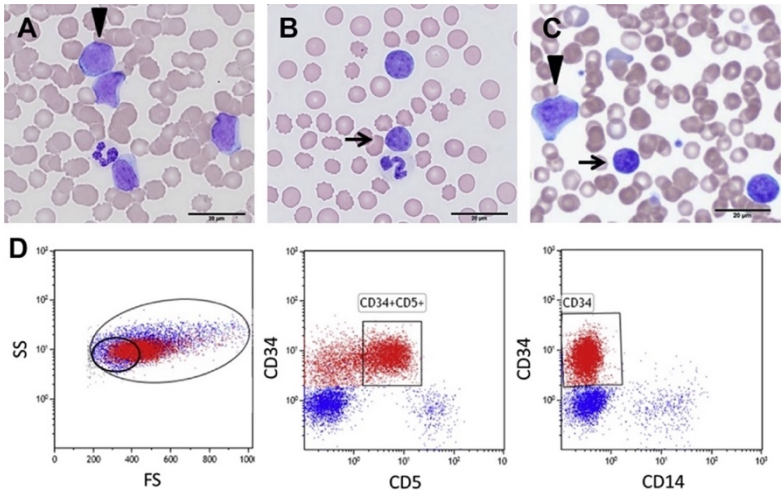


Figure 16: Peripheral blood cytology (A–C) from 3 cases of canine CD5+ CD34+ leukemia (presumed acute lymphoid leukemia) and characteristic flow cytometry findings (D).

Cells range from small with inconspicuous nucleoli (arrow) to larger with prominent nucleoli (arrowhead). The nuclear shape varies from round to irregular. The cells are CD34+ with variable amounts of CD5 expression and generally fall within the lymphocyte and monocyte scatter zones. [46]

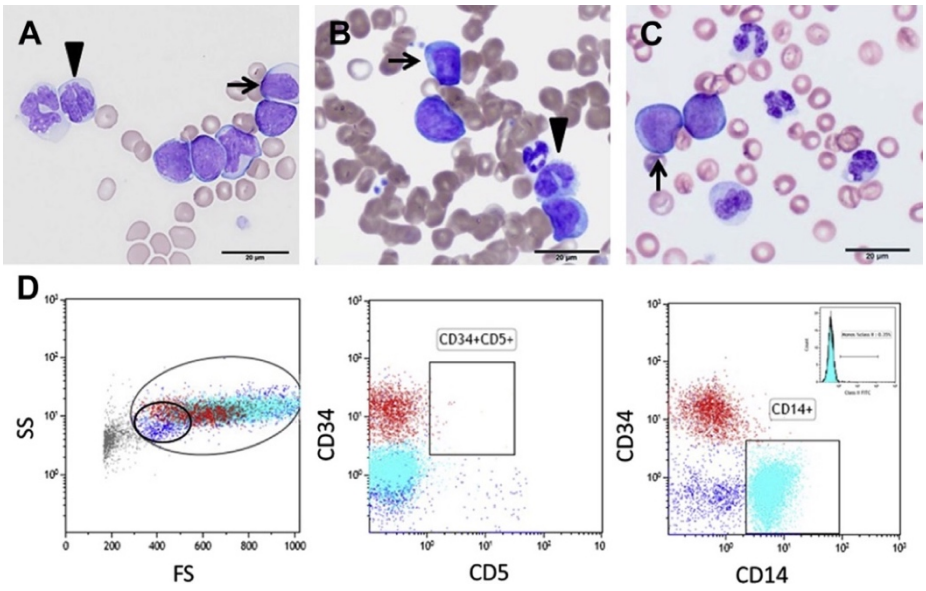


Figure 17: Peripheral blood cytology (A–C) from 3 cases of canine CD14+ CD34+ leukemia (presumed acute myeloid leukemia) and characteristic flow cytometry findings (D).

Cells tend to be intermediate-sized to large with variably apparent nucleoli. The nuclear shape varies from round (arrow) to monocytoid in appearance with indented to convoluted nuclei (arrowhead). The cells are a mixture of smaller CD341 cells (red) and larger CD141/major histocompatibility complex (MHC) class II- cells (turquoise) by flow cytometry. The inset in the right panel shows the lack of MHC class II expression contrary to what is seen on normal CD141 monocytes. [46]

The two entities can be differentiated based on CD 34 expression: CD 34 is expressed in cells originating in the bone marrow but not in lymphoid cells of another origin. In other words, CD 34 is expressed in acute lymphoid leukemia but not in stage V lymphoma. [46]

T-cell ALL was tested with different markers in a study with the result that CD34+ and CD5+ were suitable with T-cell ALL (figure 16). A similar application can be seen with acute B-cell leukemia, only with B-cell markers instead of CD5. [36]

In the case of acute myeloid neoplasia CD14+and, CD34+ cells are expected. Figure 17 demonstrates such a case.

4.5 Autoimmune disease

4.5.1 Immune-mediated hemolytic anemia

In dogs and cats, immune-mediated hemolytic anemia (IMHA) is a prevalent diagnosed immune disorder.[48, 49] It is caused by the destruction of RBCs due to intra- or extravascular hemolysis and will lead to anemia and, due to the accumulation of unconjugated bilirubin, prehepatic icterus in some animals. [50]

In cats, IMHA can be secondary to viral infections like FIV or FeLV, trauma, chronic renal failure, or blood parasite. In dogs, IMHA is often considered a primary disease but similar etiologies may be suspected as in cats (parasites, or other insulting agents against the immune system). [48] Young to middle-aged dogs are mainly affected and some breeds are more prone to get IMHA like Cocker Spaniel, Poodles, etc., which suggests a genetic implication [51]

Immune-mediated nature implies that antibodies bind to the surface of erythrocytes. Flow cytometry is applied to detect IgM and IgG on the surface of RBCs and to measure the mean fluorescence intensity (MFI).

It can be utilized for the early detection and monitoring of the treatment response in cats with IMHA. [48] In a study, including cats with- and without anemia, flow cytometry and the Coombs ‘test were performed and compared. As visible in Figure 18, flow cytometry could detect IgG and IgM on RBCs. [48]

Out of fourteen anemic cats, with the Coombs´test all of them were negative for IMHA, while FC could find IgM and IgG on RBCs in seven cats, that were in the early stage of IMHA. [48] The main advantages of flow cytometry in contrary to the Coombs´test are that fewer blood samples are needed, we can monitor the treatment of IMHA patients, and it can be used as an early diagnostic tool. [48] Considering that the diagnosis of IMHA in cats is less straightforward than in dogs owing to the indiscriminate spherocyte appearance flow cytometry appears to be an appealing diagnostic method to screen this disease.

In dogs, a similar study was performed where FC and the direct antiglobulin test (DAT) were compared to detect IgM and IgG in animals with IMHA. [49]

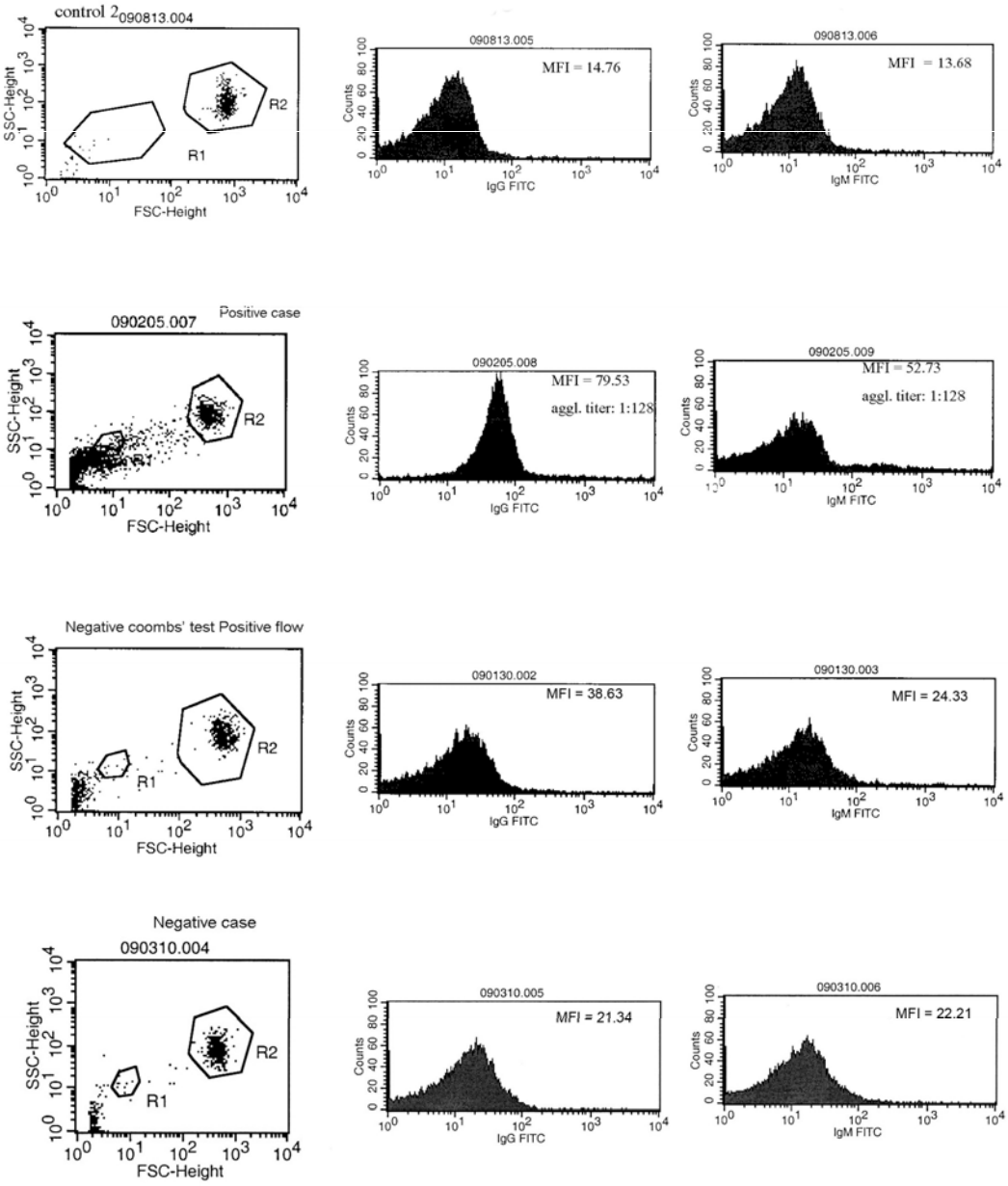


Figure 18: Flow cytometry evaluation of RBCs from healthy, non-IMHA, and IMHA cats after staining with IgG and IgM-specific secondary reagent.

FSC/SSC dot-plots and fluorescence histogram are shown for one healthy control cat, one non-IMHA anemic cat, and five IMHA anemic cats after incubation with goat anti-cat IgG (1:30) and goat anti-cat IgM (1:30). Histogram of fluorescence profiles were generated after gating on RBC in FSC/SSC dot-plots (region R2). Mean fluorescence intensities (MFI) are noted within each histogram; in anemic cats agglutination titer (AT) is also shown. [48]

While the DAT is a commonly used method to detect immunoglobulins where we can see agglutination of erythrocytes, FC can evaluate the size of the cells, their complexity, and the amount of surface fluorescence. It is a quick method that only needs a small number of samples. [49]

As above in cats, dogs with- and without anemia participated in the study. In all healthy dogs, both tests were negative for erythrocyte-bound immunoglobulins. 92% of sensitivity with FC for the detection of erythrocyte IgG, while only 53% with the DAT test was obtained, and specificity reached 100% in both tests.

Early and accurate diagnosis of IMHA is of utmost importance to start proper treatment. With flow cytometry, such a diagnosis is possible. Compared to the DAT test, it is a quick and sensitive method to evaluate erythrocyte-bound immunoglobulins. [49]

4.5.2 Immune-mediated thrombocytopenia

A somewhat prevalent cause of bleeding in small animals, particularly in dogs, is immune-mediated thrombocytopenia (ITP). [51] It is a result of increased platelet destruction and decreased platelet reproduction due to antibody and cell-mediated destruction of platelets and megakaryocytes.[51, 52] It can be of primary, an autoimmune disease, or secondary origin, like medications, neoplasms, parasites, etc. [51]

It is a primary disease of middle-aged female dogs, while in cats it is secondary and can occur at any age. Petechial hemorrhages on the ventral trunk and mucus membranes can indicate ITP. Prior to flow cytometry, ITP was often diagnosed by excluding all secondary causes.

With flow cytometry, anti-platelet antibodies can be detected and help the diagnosis. [51] The size of the platelets, but also micro aggregates, and surface granularity can be analyzed by the flow cytometer. CD41 and CD61 can be used to evaluate the activation status of platelets. For the diagnosis of ITP, FC can detect platelet-bound IgG on the surface of the platelets. With this method, certain advantages exist including the ability to quantify antibody binding at the level of each platelet individually and the assessment of small fluorescence values that are invisible to the naked eye. However, the sensitivity and specificity of the currently available methods are low and therefore clinical application is pending [53]

4.6 Functional essays

4.6.1 Platelet function analysis

Platelet function defects are rare entities and their diagnosis is often difficult. Flow cytometry may be applied along with other modalities to detect functional abnormalities. In order to assess their function platelets should be activated similarly to other platelet function evaluations. However, flow cytometry may assess the expression of surface markers quantitatively unlike currently available commercial methods (mostly measuring platelet plug formation time), and may provide information about the exact nature of platelet function defects in one essay. However, investigations are hindered by the lack of available cross-reactive MAbs for veterinary species. [12]

4.6.2 Neutrophil function tests

Studying neutrophil function classically requires various essays performed on cells harvested from the animal and performed parallelly. They are also work-intensive. Flow cytometric methods use much fewer cells (50-100X).
Surface adhesion testing of neutrophil granulocytes is carried out by measuring the expression of adhesion molecules on neutrophil cells.
Phagocyting capabilities may be essayed by fluorescently labeled material that neutrophil cells may phagocytize (e.g., bacteria). An essay has been developed for veterinary medicine not only analyses phagocytes function but with clever fluorochrome use the oxidative burst of neutrophil cells may be assessed. [12]

5 Conclusion

Flow cytometers were previously applied only in research, but nowadays they are increasingly user-friendly and are being used for the diagnosis and monitoring of clinical patients by veterinarians. [5, 31]

With immunophenotyping, the application of cluster of differentiation, gating strategy, FSC, and SSC, and multicolor approach, different cell populations can be identified and neoplastic disease can be classified and staged such as lymphoproliferative disease, B-cell and T-cells lymphomas, myeloproliferative disease, acute and chronic leukemia, immune-mediated hemolytic anemia and immune-mediated thrombocytopenia.

As mentioned above, the main advantage of flow cytometry is the noninvasive sample collection. An FNA sample from a mass or a lymph node in combination with immunophenotyping can give an accurate and quick diagnosis. Also, a wide variety of samples can be used and identify the phenotypic pattern of hematopoietic malignancies. [31]

In the case studies, flow cytometry immunophenotyping was applied for the diagnosis and compared with other techniques such as the direct antiglobulin test. As a result, in all cases, a quick and accurate diagnosis could be achieved. That doesn’t mean that FC can replace conventional clinical diagnostic methods like physical examinations, biochemistry, cytology, and diagnostic imaging, but it allows us to confirm our diagnosis and even monitor the therapy of the clinical patients.

To better describe malignant cells, more and more markers are being added. The rate of progression, the type of disease, and its stage are better defined due to the automated analytical tools applied to multiparameter datasets. Our understanding of fundamental and translational research will undoubtedly increase because of the use of computers and algorithms in conjunction with highly skilled operators and instrument standardization. [54]

6 Summary

The flow cytometer has quickly become an indispensable tool for medical research, particularly in the fields of immunology and hematology. Both, the physical characteristics and proteins, expressed by individual cells can be analyzed by flow cytometry, based on the detection of light scatter and fluorescent markers when a laser beam strikes a cell preparation. The technology has been swiftly adopted by manufacturers of hematology analyzers and transfigured into a platform that can enumerate erythrocytes, leukocytes, and platelets, and differentiate white cells. In comparison to hematology analyzers, dedicated flow cytometers incorporate several laser sources, with greater optical capacity, and more flexible output.

The adaptability and precision of flow cytometry make it a potent tool for detecting and tracking a wide range of disorders. [4]

When paired with other clinical laboratory techniques, flow cytometric analysis will likely be essential for a quick and objective evaluation of the cell surface and other intracellular properties. The creation of fluorochromes for multi-parametric analysis and the therapeutic usage of new clusters of monoclonal antibodies are both of great interest as flow cytometers become more user-friendly. [8]

The advantages and prospective applications of flow cytometry in small animal veterinary medicine are virtually limitless as technology advances. [5]

For the purpose of identifying, counting, and evaluating functions in particular cell subpopulations, flow cytometry is a laser-based method. In-office hematology analyzers, accurate evaluation of immune system cells, antibodies on RBCs and platelets, erythrocyte parasite identification, evaluation of platelet function, characterization of bone marrow cell types, immunophenotyping of cancer cells, and pharmacodynamic monitoring are some of the most frequently used applications in small animal medicine. The potential advantages and uses of flow cytometry in small animal veterinary treatment are essentially limitless as technology develops and becomes more approachable. [2]

7 Bibliography

1. Kalina T, Abraham RS, Rizzi M, van der Burg M (2020) Editorial: Application of Cytometry in Primary Immunodeficiencies. Front Immunol 11:463. https://doi.org/10.3389/FIMMU.2020.00463/BIBTEX
2. Archer T, Mackin A, Pinchuk LM (2014) Applications of Flow Cytometry in Veterinary Research and Small Animal Clinical Practice Article in Journal of Applied Research in Veterinary Medicine
3. Alfonso AF, Al-Rubeai M (2019) Flow cytometry. Comprehensive Biotechnology 541–560. https://doi.org/10.1016/B978-0-444-64046-8.00048-3
4. Tarrant JM (2005) The role of flow cytometry in companion animal diagnostic medicine. The Veterinary Journal 170:278–288. https://doi.org/10.1016/J.TVJL.2004.06.010
5. Thomason J, Archer T, Mackin A, Stokes J, Pinchuk L (2014) Applications of Flow Cytometry in Veterinary Research and Small Animal Clinical Practice. J Vet Med Res 1:1004
6. Reggeti F, Bienzle D (2011) Flow cytometry in veterinary oncology. Vet Pathol 48:223–35. https://doi.org/10.1177/0300985810379435
7. Meichner K, Stokol | Tracy, Tarigo | Jaime, Avery A, Burkhard MJ, Comazzi S, Fogle J, Devorah |, Stowe M, Rütgen B, Seelig D, Wasserkrug-Naor A, Vernau W, Dorothee Bienzle | (2020) O R I G I N A L A R T I C L E Multicenter flow cytometry proficiency testing of canine blood and lymph node samples. https://doi.org/10.1111/vcp.12843
8. Molla D, Tekle Z, Ayisheshim A, Kebede D, Wondimu A, Ahmed WM (2019) Flow Cytometry and Its Diagnostic Application in Animal Health: A Review. Glob Vet 21:1–07. https://doi.org/10.5829/idosi.gv.2019.01.07
9. Dottorato S di, Martini Matr VR (2013) CLINICAL PATHOLOGICAL FEATURES OF CANINE HEMATOPOIETIC NEOPLASMS ASSESSED VIA FLOW CYTOMETRY. https://doi.org/10.13130/MARTINI-VALERIA_PHD2014-02-11
10. Wilkerson MJ (2012) Principles and Applications of Flow Cytometry and Cell Sorting in Companion Animal Medicine. Veterinary Clinics of North America - Small Animal Practice 42:53–71
11. Reggeti F, Bienzle D (2011) Flow cytometry in veterinary oncology. Vet Pathol 48:223–235. https://doi.org/10.1177/0300985810379435
12. [PDF] Schalm's Veterinary Hematology by Marjory B. Brooks, Kendal E. Harr, Davis M. Seelig, K. Jane Wardrop, Douglas J. Weiss | Perlego. https://www.perlego.com/book/3271849/schalms-veterinary-hematology-pdf. Accessed 8 Sep 2022
13. Jahan-Tigh RR, Ryan C, Obermoser G, Schwarzenberger K (2012) Flow Cytometry. Journal of Investigative Dermatology 1. https://doi.org/10.1038/jid.2012.282
14. Jahan-Tigh RR, Ryan C, Obermoser G, Schwarzenberger K (2012) Flow Cytometry. Journal of Investigative Dermatology 1. https://doi.org/10.1038/jid.2012.282
15. Hunka J, Riley JT, Debes GF (2020) Approaches to overcome flow cytometry limitations in the analysis of cells from veterinary relevant species. BMC Vet Res 16:. https://doi.org/10.1186/s12917-020-02299-2
16. BD Spectrum Viewer. https://www.bdbiosciences.com/en-eu/resources/bd-spectrum-viewer. Accessed 18 Nov 2022

17. Betters, PhD, RN DM (2015) Use of Flow Cytometry in Clinical Practice. J Adv Pract Oncol 6:435. https://doi.org/10.6004/JADPRO.2015.6.5.4
18. Johnson LA (1995) Sex preselection by flow cytometric separation of X and Y chromosome-bearing sperm based on DNA difference: a review. Reprod Fertil Dev 7:893–903. https://doi.org/10.1071/RD9950893
19. Hodne K, Weltzien FA (2015) Single-cell isolation and gene analysis: Pitfalls and possibilities. Int J Mol Sci 16:26832–26849. https://doi.org/10.3390/IJMS161125996
20. Gibson D, Aubert I, Woods JP, Abrams-Ogg A, Kruth S, Wood RD, Bienzle D (2004) Flow Cytometric Immunophenotype of Canine Lymph Node Aspirates. J Vet Intern Med 18:710–717. https://doi.org/10.1111/J.1939-1676.2004.TB02610.X
21. Jalla S, Sazawal S, Deb S, Black RE, Das SN, Sarkar A, Bhan MK (2004) Enumeration of lymphocyte subsets using flow cytometry: Effect of storage before and after staining in a developing country setting. Indian Journal of Clinical Biochemistry 2004 19:2 19:95–99. https://doi.org/10.1007/BF02894264
22. Khowawisetsut L, Sukapirom K, Pattanapanyasat K Data analysis and presentation in flow cytometry. https://doi.org/10.2306/scienceasia1513-1874.2018.44S.019
23. Misha Rahman B, Lane A, Swindell A, Bartram S Introduction to Flow Cytometry
24. Owens MA, Vall HG, Hurley AA, Wormsley SB (2000) Validation and quality control of immunophenotyping in clinical flow cytometry. J Immunol Methods 243:33–50. https://doi.org/10.1016/S0022-1759(00)00226-X
25. Scheuermann RH, Bui J, Wang HY, Qian Y (2017) Automated Analysis of Clinical Flow Cytometry Data: A Chronic Lymphocytic Leukemia Illustration. Clin Lab Med 37:931–944. https://doi.org/10.1016/J.CLL.2017.07.011
26. Comazzi S, Avery PR, Garden OA, Riondato F, Rütgen B, Vernau W (2017) European canine lymphoma network consensus recommendations for reporting flow cytometry in canine hematopoietic neoplasms. Cytometry B Clin Cytom 92:411–419. https://doi.org/10.1002/CYTO.B.21382
27. Herold NC, Mitra P (2022) Immunophenotyping. Childhood Leukemias, Third Edition 72–112. https://doi.org/10.1017/CBO9780511977633.005
28. Aniolek O, Gajewski Z, Gizinski S (2014) Application of flow cytometry in diagnosing lymphomas in dogs and cats. Central European Journal of Immunology 39:327–330. https://doi.org/10.5114/CEJI.2014.45943
29. Marjory B. Brooks KEHDMSKJWDJW (2022) Schalm's Veterinary Hematology. In: 7th ed. Wiley-Blackwell, pp 492–493
30. Valli VE, Myint M, Barthel A, Bienzle D, Caswell J, Colbatzky F, Durham A, Ehrhart EJ, Johnson Y, Jones C, Kiupel M, Labelle P, Lester S, Miller M, Moore P, Moroff S, Roccabianca P, Ramos-Vara J, Ross A, Scase T, Tvedten H, Vernau W (2011) Classification of canine malignant lymphomas according to the world health organization criteria. Vet Pathol 48:198–211. https://doi.org/10.1177/0300985810379428/ASSET/IMAGES/LARGE/10.1177_0300985810379428-FIG2.JPEG
31. Guzera M, Cian F, Leo C, Winnicka A, Archer J (2016) The use of flow cytometry for immunophenotyping lymphoproliferative disorders in cats: a retrospective study of 19 cases. Vet Comp Oncol 14:40–51. https://doi.org/10.1111/VCO.12098

32. Calvert J (2019) B-cell chronic lymphocytic leukemia in a miniature American Eskimo dog. The Canadian Veterinary Journal 60:1353
33. Suter SE, Fogle J, Miglio A, Kisseberth W, Riondato F, Comazzi S (2021) Flow Cytometry in the Diagnosis of Canine B-Cell Lymphoma. Frontiers in Veterinary Science | www.frontiersin.org 1:. https://doi.org/10.3389/fvets.2021.600986
34. Comazzi S, Gelain ME (2011) Use of flow cytometric immunophenotyping to refine the cytological diagnosis of canine lymphoma. The Veterinary Journal 188:149–155. https://doi.org/10.1016/J.TVJL.2010.03.011
35. Vernau W, Moore PF (1999) An immunophenotypic study of canine leukemias and preliminary assessment of clonality by polymerase chain reaction. Vet Immunol Immunopathol 69:145–164. https://doi.org/10.1016/S0165-2427(99)00051-3
36. Aresu L, Aricò A, Ferraresso S, Martini V, Comazzi S, Riondato F, Giantin M, Dacasto M, Guadagnin E, Frayssinet P, Rouquet N, Drigo M, Marconato L (2014) Minimal residual disease detection by flow cytometry and PARR in lymph node, peripheral blood and bone marrow, following treatment of dogs with diffuse large B-cell lymphoma. The Veterinary Journal 200:318–324. https://doi.org/10.1016/J.TVJL.2014.03.006
37. Rütgen BC, Baszler E, Weingand N, Wolfesberger B, Baumgartner D, Hammer SE, Groiss S, Fuchs-Baumgartinger A, Saalmüller A, Schwendenwein I (2021) Composition of lymphocyte subpopulations in normal and mildly reactive peripheral lymph nodes in cats. https://doi.org/101177/1098612X211005310 24:77–90. https://doi.org/10.1177/1098612X211005310
38. Platt R, Ng T, Glover S, Roof M, Kimura K, Uk A Canine Peripheral Blood Lymphocyte Phenotyping by 7-Color Multiparameter Flow Cytometry CORE View metadata, citation and similar papers at core
39. Hughes KL, Ehrhart EJ, Rout ED, Harris LJ, Fernandez M, Yoshimoto JA, Dossey J, Kuzmik AR, Avery PR, Avery AC Special Issue: Diagnostic Veterinary Oncologic Pathology-Original Article Diffuse Small B-Cell Lymphoma: A High-Grade Malignancy. https://doi.org/10.1177/0300985820985221
40. Modiano JF, Breen M, Burnett RC, Parker HG, Inusah S, Thomas R, Avery PR, Lindblad-Toh K, Ostrander EA, Cutter GC, Avery AC (2005) Distinct B-Cell and T-Cell Lymphoproliferative Disease Prevalence among Dog Breeds Indicates Heritable Risk. Cancer Res 65:5654–5661. https://doi.org/10.1158/0008-5472.CAN-04-4613
41. Riondato F, Comazzi S (2021) Flow Cytometry in the Diagnosis of Canine B-Cell Lymphoma. Front Vet Sci 8:253. https://doi.org/10.3389/FVETS.2021.600986/BIBTEX
42. Purzycka K, Peters LM, Desmas I, Davies O, Chang YM, Lara-Garcia A (2020) Clinicopathological characteristics and prognostic factors for canine multicentric non-indolent T-cell lymphoma: 107 cases. Vet Comp Oncol 18:656–663. https://doi.org/10.1111/VCO.12589
43. Comazzi S, Riondato F (2021) Flow Cytometry in the Diagnosis of Canine T-Cell Lymphoma. Front Vet Sci 8:. https://doi.org/10.3389/FVETS.2021.600963
44. Ferrari A, Cozzi M, Aresu L, Martini V (2021) Tumor staging in a Beagle dog with concomitant large B-cell lymphoma and T-cell acute lymphoblastic leukemia. Journal of Veterinary Diagnostic Investigation 33:792–796.

https://doi.org/10.1177/10406387211011024/ASSET/IMAGES/LARGE/10.1177_10406387211011024-FIG2.JPEG
45. Rout ED, Moore AR, Burnett RC, Labadie JD, Hughes KL, Navin PA, Yoshimoto JA, Avery PR, Avery AC (2020) Polyclonal B-cell lymphocytosis in English bulldogs. J Vet Intern Med 34:2622–2635. https://doi.org/10.1111/JVIM.15913
46. Rout ED, Avery PR (2017) Lymphoid Neoplasia Correlations Between Morphology and Flow Cytometry. Veterinary Clinics of NA: Small Animal Practice 47:53–70. https://doi.org/10.1016/j.cvsm.2016.07.004
47. Ouyang G, Xu Z, Jiang D, Zhu H, Wang Y, Wu W, Sun Y, Sheng L, Xu K, Lou Y, Mu Q, Zhang Y, Wu N, Cheng J, Duan S (2019) Clinically useful flow cytometry approach to identify immunophenotype in acute leukemia. J Int Med Res 47:1483. https://doi.org/10.1177/0300060518819637
48. Rungsipipat A, Pusoonthornthum R Application of Flow Cytometry for Early Diagnosis and Monitoring in Cats with Immune-mediated Hemolytic Anemia
49. Quigley KA, Chelack BJ, Haines DM, Jackson ML (2016) Application of a Direct Flow Cytometric Erythrocyte Immunofluorescence Assay in Dogs with Immune-Mediated Hemolytic Anemia and Comparison to the Direct Antiglobulin Test. https://doi.org/101177/104063870101300403 13:297–300. https://doi.org/10.1177/104063870101300403
50. Swann JW, Skelly BJ (2016) Canine autoimmune hemolytic anemia: management challenges. Veterinary Medicine : Research and Reports 7:101. https://doi.org/10.2147/VMRR.S81869
51. Mackin BVSc BVMS MVS DVSc FANZCVSc DipACVIM A IMMUNE-MEDIATED HEMOLYTIC ANEMIA: PATHOPHYSIOLOGY AND DIAGNOSIS
52. Allen EC, Tarigo JL, LeVine DN, Barber JP, Brainard BM (2021) Platelet number and function in response to a single intravenous dose of vincristine. J Vet Intern Med 35:1754–1762. https://doi.org/10.1111/JVIM.16169
53. Cortese L, Christopherson PW, Pelagalli A (2020) Platelet Function and Therapeutic Applications in Dogs: Current Status and Future Prospects. Animals 2020, Vol 10, Page 201 10:201. https://doi.org/10.3390/ANI10020201
54. Lugli E, Roederer M, Cossarizza A (2010) Data analysis in flow cytometry: The future just started. Cytometry Part A 77A:705–713. https://doi.org/10.1002/CYTO.A.20901

8 Acknowledgment

I would like to thank my parents, without them I would not be, where I am now. I also want to thank my brother, for always having my back and supporting me, as my partner.

Also thanks to my friends, who made the past years in Budapest unforgettable.

HuVetA

ELECTRONIC LICENSE AGREEMENT AND COPYRIGHT DECLARATION*

Name: JOHANNA FRANK

Contact information (e-mail): johanna.frank137@gmail.com

Title of document (to be uploaded): FLOW CYTOMETRY IN VETERINARY MEDICINE

Publication data of document: 18.11.2022

Number of files submitted: 1

By accepting the present agreement the author or copyright owner grants non-exclusive license to HuVetA over the above mentioned document (including its abstract) to be converted to copy protected PDF format without changing its content, in order to archive, reproduce, and make accessible under the conditions specified below.

The author agrees that HuVetA may store more than one copy (accessible only to HuVetA administrators) of the licensed document exclusively for purposes of secure storage and backup, if necessary.

You state that the submission is your original work, and that you have the right to grant the rights contained in this license. You also state that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the document has parts which you are not the copyright owner of, you have to indicate that you have obtained unrestricted permission from the copyright owner to grant the rights required by this Agreement, and that any such third-party owned material is clearly identified and acknowledged within the text of the licensed document.

The copyright owner defines the scope of access to the document stored in HuVetA as follows (**mark the appropriate box with an X)**:

- [X] I grant unlimited online access,
- [ ] I grant access only through the intranet (IP range) of the University of Veterinary Medicine,
- [ ] I grant access only on one dedicated computer at the Ferenc Hutÿra Library,
- [ ] I grant unlimited online access only to the bibliographic data and abstract of the document.

Please, define the **in-house accessibility of the document** by marking the below box with an **X**:

[X] I grant in-house access (namely, reading the hard copy version of the document) at the Library.

If the preparation of the document to be uploaded was supported or sponsored by a firm or an organization, you also declare that you are entitled to sign the present Agreement concerning the document.

The operators of HuVetA do not assume any legal liability or responsibility towards the author/copyright holder/organizations in case somebody uses the material legally uploaded to HuVetA in a way that is unlawful.

Date: Budapest, 18.day 11.month 2022.year

Author/copyright owner signature

HuVetA Magyar Állatorvos-tudományi Archívum – Hungarian Veterinary Archive *is an online veterinary repository operated by the Ferenc Hutÿra Library, Archives and Museum. It is an electronic knowledge base which aims to collect, organize, store documents regarding Hungarian veterinary science and history, and make them searchable and accessible in line with current legal requirements and regulations.*

HuVetA relies on the latest technology in order to provide easy searchability (by search engines, as well) and access to the full text document, whenever possible.
Based on the above, HuVetA aims to:

- *increase awareness of Hungarian veterinary science not only in Hungary, but also internationally;*
- *increase citation numbers of publications authored by Hungarian veterinarians, thus improve the impact factor of Hungarian veterinary journals;*
- *present the knowledge base of the University of Veterinary Medicine Budapest and its partners in a focussed way in order to improve the prestige of the Hungarian veterinary profession, and the competitiveness of the organizations in question;*
- *facilitate professional relations and collaboration;*
- *support open access.*

I hereby confirm that I am familiar with the content of the thesis entitled

Flow cytometry in veterinary medicine – literature review

written by

Johanna Frank

which I deem suitable for submission and defence.

Date: Budapest, 17 November 2022

.....

Supervisor name and signature

Department of Clinical Pathology and Oncology

UNIVERSITY OF VETERINARY MEDICINE, BUDAPEST
founded in 1787, EU-accredited since 1995

INTERNATIONAL STUDY PROGRAMS
secretary, student@univet.hu

Thesis progress report for veterinary students

Name of student: JOHANNA FRANK

Neptun code of the student: ARYEB2

Name and title of the supervisor: MÁRTON MARIALIGETI, DEPT VETERINARIAN

Department: DEPARTMENT OF CLINICAL PATHOLOGY AND ONCOLOGY

Thesis title: FLOW CYTOMETRY IN VETERINARY MEDICINE – A LITERATURE REVIEW

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	02	14	–	
2.	2022	03	28	–	
3.	2022	04	25	–	
4.	2022	05	16	–	
5.	2022	06	27	–	

Grade achieved at the end of the first semester: good(4)

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	09	22	–	
2.	2022	10	07	–	
3.	2022	10	21	proofreading	
4.	2022	11	14	finalization	

UNIVERSITY OF VETERINARY MEDICINE, BUDAPEST
INTERNATIONAL STUDY PROGRAMS
founded in 1787, EU-accredited since 1995
secretary, student@univet.hu

2022 11 17

Grade achieved at the end of the second semester: ..... good (4) .....

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

.....
signature of the supervisor

Signature of the student: .....

Signature of the secretary of the department: .....

Date of handing the thesis in..... 18. 11. 2022 .....