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Approaches to overcome flow cytometry limitations in the analysis of cells from veterinary relevant species

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Abbreviations

BV	Brilliant Violet
CD	Cluster of differentiation
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FITC	Fluorescein
FSC	Forward scatter
Nm	Nanometers
PE	Phycoerythrin
PECy7	Phycoerythrin-cyanine-7
SSC	Side scatter
UV	Ultraviolet

1 Introduction

The domesticated sheep *(Ovis aries)* has co-evolved alongside humans for thousands of years as one of the first domesticated species[1]. The sheep is not only a vital part of agriculture and economic systems but also serves as an important research model. The size, docile nature and the possibility of lymphatic cannulation makes sheep an ideal model for the investigation of its immune system. Research in sheep has led to milestones in knowledge about the sheep immune system and the understanding of immune response mechanisms for the benefit of general animal and human physiology and disease [2-5]. Furthermore, knowledge gained in large animals like sheep can in certain instances be more effectively translated into successful clinical outcomes in humans [5].

Flow cytometry is an important tool with applications in various disciplines and clinical settings and is used by laboratories across the world. It is a tool for advanced cellular phenotyping using fluorochrome (color) labeled antibodies specific to surface and intracellular proteins. The fluorochromes can be excited by lasers and captured by detectors to identify protein expression at the single cell level. Due to the popularity of flow cytometry in rodents and humans, a wide range of antibodies and other reagents are commercially available with easy to follow protocols [4-6]. For these species, an extensive assortment of different antibodies comes in a large variety of color choices by commercial vendors at competitive prices. Thereby enabling the composition of state-of-the-art flow cytometry panels to characterize cells through their expression of multiple surface and intracellular proteins [7]. These multiparameter or multicolor flow cytometry panels allow for fast and simultaneous analysis of multiple parameters of cells to easily characterize and study single cells in a mixed population or in a larger context of functional interactions between cells [8-13].

Our laboratory employs the lymphatic cannulation model in sheep to study skin specific lymphocyte subsets. This model is well established and has been utilized in several important lymphocyte migration studies. But given that sheep are used far less frequently than other model organisms the sheep-specific flow cytometry reagents are limited[5, 6]. In our work with sheep we faced different obstacles in the analysis of lymphocytes using complex flow cytometry panels because there is

a partial lack of commercially available monoclonal antibodies, including some to very basic markers like the B cell specific antigen CD19. If monoclonal antibodies exist, they are often only available purified or in limited color choices. These problems arise not only with sheep, but also when using species other than humans and mice. For example, studies of immune responses in large domestic animals comprise less than 5% of the publications in major immunology journals [14]. The studies of the immune system for veterinary relevant species are restricted mainly by limited monoclonal antibody reagent availability for research [15-19]. This makes it difficult to build state of the art panels for multiparameter analysis in veterinary clinical medicine and research, a void which this thesis work addresses. The results are part of this work and published in *BMC Veterinary Research, Hunka J. et al. 2020*. In this publication we present approaches to overcome these limitations and help to expand the use of multiparameter flow cytometry in veterinary species. Using these approaches we can characterize immune cell subsets and lay the basis for a better understanding of the immune system of veterinary species to enhance treatment and prevention of diseases in both animals and humans [13, 15].

2 Fundamentals

2.1 Flow cytometry

2.1.1 A brief history of flow cytometry

In 1965, Mack J. Fulwyler was the first person to build a flow cytometer to separate cells by volume [20]. He wanted to counter the idea that there are two populations of red blood cells and was inspired by the technology of ink jet printers, which was invented by Richard Sweet [21] years before. Shortly after, Herzenberg et al were the first to describe fluorescence-based flow cytometry at Stanford University [22-24]. They combined Fulwyler's technology with fluorescently labeled monoclonal antibodies [25] and sorted the first cells based on fluorescence. Flow cytometry became commercial in the 1970s when Becton Dickinson Immunocytometry Systems starting manufacturing flow cytometry instruments [24]. Flow cytometry quickly became indispensable in many laboratories across the world as it enabled the fast and simultaneous analysis of multiple different parameters on a single cell level using commercially available fluorochrome labeled antibodies [21, 26, 27]. There are currently over 10,000 different types of flow cytometers on the market [8, 28] and with technological progress flow cytometry has become easier to use and more accessible over the last few decades, and is a relevant part of veterinary research and veterinary clinical medicine [29].

2.1.2 Basic principles of flow cytometry

Visible light is defined as an electromagnetic radiation with wavelengths between ~400 and 700 nm [30]. In a flow cytometer the light source is a laser, which emits light at discrete wavelengths in the ultraviolet (UV) and visible light spectra and is used to analyze heterogenous cell samples. Flow cytometers are designed so that one cell at a time passes through the laser beam. Small particles, like cells, scatter light when they encounter the laser, which is translated as forward scatter (FSC; light scattered at small angles 0.5-5°), which shows the size of the cell, and side scatter (SSC; light scattered at larger angles 15-150°), indicating its granularity. Based on the combination of forward and side scatter properties, immune cell populations can be distinguished by their size and granularity, similar to microscopy [28, 30-32]. Figure 1 depicts a gating strategy for discriminating

lymphocytes, monocytes and granulocytes based on side and forward scatter properties.

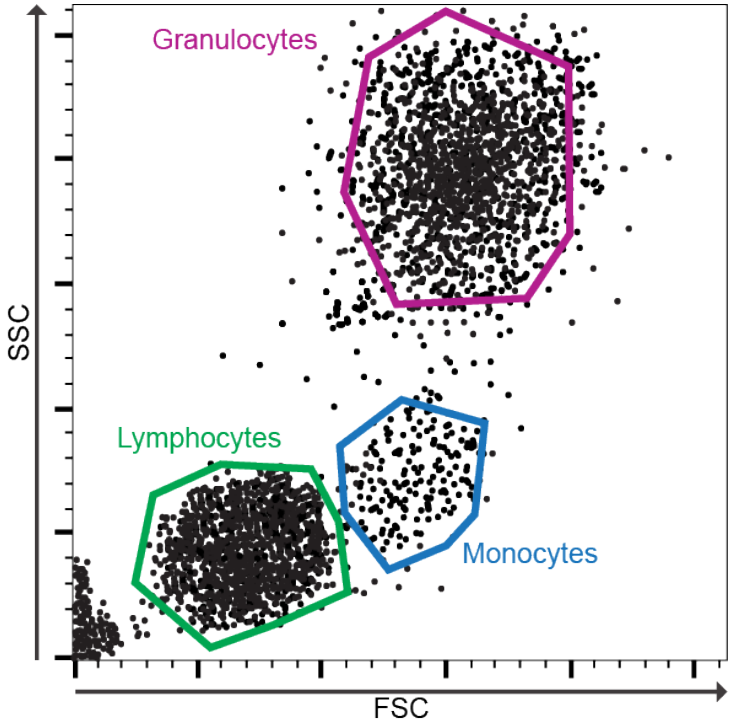

Figure 1: Discrimination of immune cells based on forward and side scatter properties
Simplified depiction of leukocytes analyzed by flow cytometry. Each dot represents one cell. Three different cell population can be identified base on size (FSC) and granularity (SSC). Lymphocytes (green, smallest and few/none granules), monocytes (blue, larger and higher granularity than lymphocytes) and granulocytes (orange, highest amount of granularity).

Furthermore, cells can be distinguished based on the expression of surface or intracellular proteins to which fluorochrome labeled antibodies bind, first described by Coons et al [33, 34]. Fluorochromes emit light when excited by the laser, based on the physical principle of quantum electrodynamic. It states that, when a particle is excited by light of a specific wavelengths the electrons absorbs the energy and the particle goes from its ground state of energy to a higher state of energy. When the electrons return to their ground state, from the higher state back to the lower state of energy, a photon of light is emitted. This process is called fluorescence, hence fluorescence activated cell sorting or FACS [30, 35]. A fluorochrome is excited by light of a certain wavelengths and in response emits light at different, longer wavelengths (depicted in Figure 2).

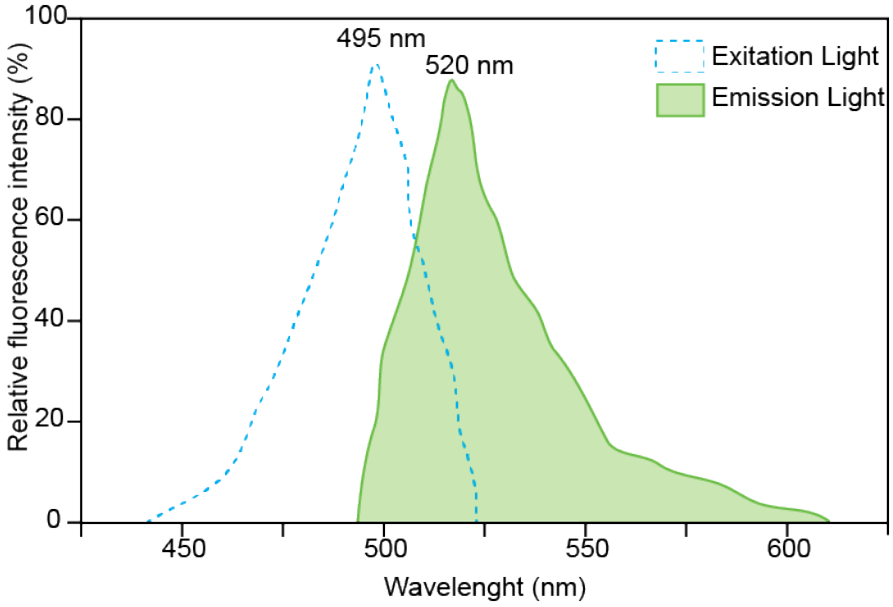

Figure 2: Excitation and emission spectrum of fluorescein (FITC)
Schematic representation of the excitation and emission spectrum of FITC. The blue dotted line depicts the spectrum in which a FITC molecule can be excited, the maximum peak of optimal excitation for FITC is at 495nm. Blue lasers in flow cytometers are usually 488nm, which will excite the FITC molecule that will then emit light at a range of higher wavelengths, peaking at 520nm, depicted by the filled green line. Detectors in the flow cytometer will capture the light emitted at and around 520nm as a positive signal from FITC excitation. Adapted from [36].

This emitted light can be detected by optical filter and photodetectors, which are part of the optics of a flow cytometer. The components of a flow cytometer are more extensively discussed in the next section.

2.1.3 Setup of a flow cytometer

A flow cytometer has three main components: electronics, fluidics and optics systems [37]. The discussion of the electronics system is beyond the scope of this thesis and more detailed guidelines to flow cytometry can be found elsewhere [8, 9, 31, 38]. The fluidics system uses a principle called hydrodynamic focusing to generate a stream of sheath fluid (the liquid that runs through a flow cytometer) combined with the cell suspension, that is so small that one cell at a time passes through the stream and past the laser beam [27, 39]. The optics system consists of lasers, lenses and filters. The lasers of a flow cytometer emit focused light of certain wavelengths. The most common lasers used in flow cytometry are UV, violet, blue, green, yellow, and red, which are described in *Hunka et al 2020* together with their respective wavelengths. The flow cell of a flow cytometer is the interrogation point

where the single cell suspension passes between the excitatory light emitted from the laser, and the detection systems [39]. As each cell passes through the interrogation point, light from each laser is directed at the cell, and if a cell has fluorescent antibodies bound to phenotypic markers, the fluorophore will be excited and will emit light, which is then passed through dichroic mirrors and optical filters to identify the wavelength of light emitted. This information is collected by the detector system and processed by the electronics system to quantify the amount of light scatter and fluorescence detected on an individual cell basis [37]. Each flow cytometer can be designed to include multiple lasers, various filters and detectors to create instruments with increasing capabilities to detect large numbers of parameters (fluorophores).

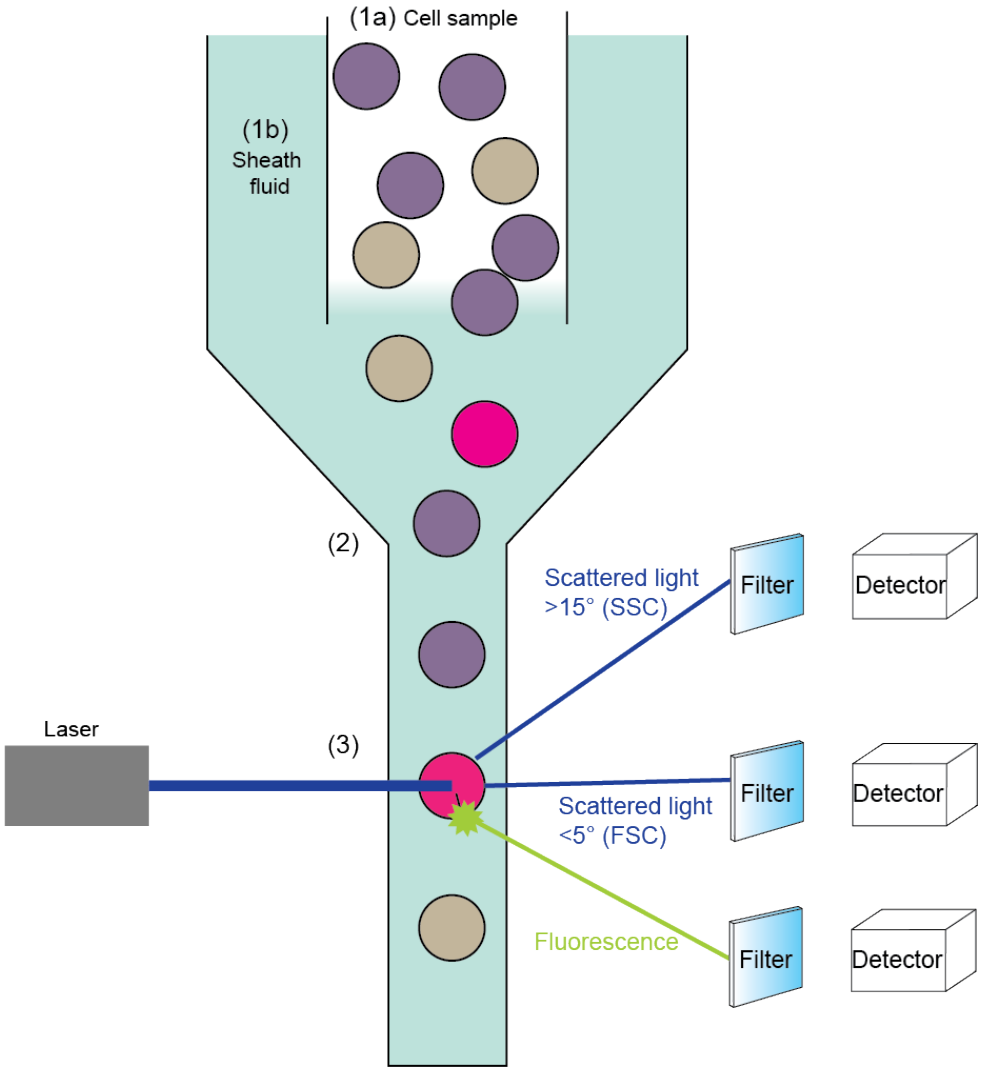

Figure 3: Setup of a flow cytometer
Depicted is the setup of the main compartments of a flow cytometer. **(1)** Fluidics system , consisting of (1a) cell sample and (1b) sheath fluid (1b). **(2)** Through

hydrodynamic focusing a single cell stream is formed. **(3)** At the interrogation point the cells encounter the excitation light of laser and activated fluorophores emit light. **(4)** The emitted light is detected by filter and detector sets. The figure shows one laser and three filter and detector sets as an example. In modern flow cytometers multiple laser, filter and detector sets are used to detect different wavelengths of emitted light.

2.2 Applications of flow cytometry in veterinary clinical medicine and research

There are countless applications for flow cytometry [21] and in veterinary clinical medicine flow cytometry is regularly used in pathology, parasitology [40], oncology [41], and pharmacology, for example when monitoring immunosuppressive drugs in dogs by observing their immune system [42, 43]. Furthermore, flow cytometry is extensively used in hematology and can be incorporated into hematology analyzers [44]. This enables its use for diagnostics such as reticulocyte counting [45], immunophenotyping of white blood cells, detection of erythrocytic parasites [46] or assessment of neutrophil function [44]. In dogs and horses, it was successfully demonstrated that flow cytometry can aid in the diagnosis of Immune-mediated hemolytic anemia (IMHA) or thrombocytopenia through analysis of erythrocytes or evaluations of platelets respectively [29, 52, 53]. When combining flow cytometry, hematology and oncology, it can be used for the study and diagnosis of lymphomas [47] and leukemias [48, 49], which is utilized by veterinary clinicians [50, 51]. Here, flow cytometry can assist with classification of lymphoma types, by evaluating lineage markers (B lymphocytes, T lymphocytes, natural killer (NK) cells, myelomonocytic cells, plasma cells) and staining for certain antigens, like CD3, CD4, CD1, CD21 and MHCII is an inherent part of this classification [49]. Furthermore, flow cytometry is often used in spermatology to test fertility and health [54, 55] or to sex sperm prior to insemination[56], which can be useful for breeding meat or dairy cows and other agricultural species.

In veterinary research, flow cytometry can be employed to measure the amount of DNA in a cell, identify cell cycle and growth, perform functional cell assays or to immunophenotype cells [49, 57, 58], which is the primary focus of my thesis work. Flow cytometry was used to establish a broader knowledge of the immune system in a large range of different veterinary species. An example is its application to better characterize lymphocyte populations in dogs. Research demonstrated the

characterization of $CD4^+$ and $CD8^+$ double positive canine T cells, which were shown to exhibit features of activated T cells [59, 60]. A lot of research was enabled through the development of monoclonal antibodies for the corresponding species [61, 62]. For example, a recent study described work developing a monoclonal antibody to bovine interleukin-17A to better study the host's defense against infections [17]. However, the development of new monoclonal antibodies for veterinary relevant species progresses slowly and lacks behind mice [63]. Furthermore, this process can be complicated, time consuming and requires resources that are not available to everyone. An easier approach to expand the reagents for flow cytometry studies is species cross-reactivity of monoclonal antibodies. This approach makes use of antibodies that target antigens of one species but show documented cross-reactivity for a different species. This is an established method and many human, mouse and other animal antibodies have been tested for species cross reactivity for veterinary species [67, 68]. For example, cattle, sheep and goat monoclonal antibodies were employed to study the immune system of the water buffalo *(Bubalus bubalis)*, where immunological studies are extremely limited, due to insufficient availability of monoclonal antibodies. [64, 65]. Another group showed the cross reactivity of human CD monoclonal antibodies to a more established species in immunological research, the sheep [66].

2.3 The relevance of the domesticated sheep *(Ovis aries)* in veterinary research and clinical medicine

Sheep have been a fundamental model for the study of the immune system [3-5]. Their docile nature and size allow for longitudinal analysis and samples from different organs and tissues like blood, lung and lymph [4]. Specifically, lymph can be obtained through lymph vessel cannulation, which is the method I have employed in my dissertation work. It has been used since the 1960s and was pioneered by scientist Bede Morris [69, 70]. It is difficult to obtain lymph samples from humans or mice. Human donors of lymph are exceedingly rare, because of ethical limitations and rodents are not an ideal model due to their size. However, for immunological studies it is preferable to analyze lymphocytes during their physiological recirculation through tissues, and this can be made possible in the sheep model [2, 71, 72]. Sheep are a strong model for certain human physiology and diseases and therefore play an important role in research. [2]. Specifically, they

are an established model for many biomedical studies like heart valve replacements [73] or orthopedic research and osteoporosis [74, 75].

In addition, sheep are certainly an important species for veterinary medicine in their own right [76]. The domesticated sheep *(Ovis aries)* was one of the first species to be domesticated and it is believed that their origin is in the mountainous regions of southwest and central Asia, 8,000-10,000 years ago [77, 78]. Humans domesticated sheep for their meat, wool and milk, and they remain an important agricultural species. In countries like New Zealand, sheep are of great economic importance [79], and they have significant representation in most agriculture systems around the world, as it is estimated that there are more than 1000 distinct sheep breeds [80] and there is a total of one billion sheep globally [81]. They are raised in different environmental conditions worldwide and in all livestock production systems [82]. Because of this, there is an immense need and responsibility for the veterinary profession to improve the knowledge and care of these animals.

2.4 Multiparameter flow cytometry and its applications

It is relatively common for flow cytometers to be capable of analyzing 20 parameters, and publications have successfully demonstrated 28 parameter panels to characterize leukocytes [83], and new machines enable the theoretical analyses of 50 parameters simultaneously [27]. In contrast, the majority of published veterinary flow cytometry papers utilizes panels with a maximum of 10 parameters [15, 29, 84-86] as the availability of monoclonal antibodies lags behind mouse and humans [5].

Multiparameter flow cytometry allows for the fast and simultaneous analysis of multiple parameters of cells to easily characterize them in a mixed population. It presents an efficient way to gain a wide range of information from each analyzed sample, even rare samples, and allows for the characterization of novel cell subsets. It can provide crucial insight into functional interactions between cells and identify phenotypically similar subsets of cells within the immensely complex immune system. This information is crucial for understanding the pathogenesis of diseases [8-13].

3 Objective

This work demonstrates different approaches to overcome limitations in flow cytometric analysis that arise when using veterinary relevant species to study their immune system. It demonstrates the importance of multiparameter flow cytometry in the analysis of cells from veterinary relevant species and can be a starting point for researchers and clinicians, who wish to expand their tool kit for flow cytometric analysis.

4 Publication

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METHODOLOGY ARTICLE Open Access

Approaches to overcome flow cytometry limitations in the analysis of cells from veterinary relevant species

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Abstract

Background: Flow cytometry is a powerful tool for the multiparameter analysis of leukocyte subsets on the single cell level. Recent advances have greatly increased the number of fluorochrome-labeled antibodies in flow cytometry. In particular, an increase in available fluorochromes with distinct excitation and emission spectra combined with novel multicolor flow cytometers with several lasers have enhanced the generation of multidimensional expression data for leukocytes and other cell types. However, these advances have mainly benefited the analysis of human or mouse cell samples given the lack of reagents for most animal species. The flow cytometric analysis of important veterinary, agricultural, wildlife, and other animal species is still hampered by several technical limitations, even though animal species other than the mouse can serve as more accurate models of specific human physiology and diseases.

Results: Here we present time-tested approaches that our laboratory regularly uses in the multiparameter flow cytometric analysis of ovine leukocytes. The discussed approaches will be applicable to the analysis of cells from most animal species and include direct modification of antibodies by covalent conjugation or Fc-directed labeling (Zenon™ technology), labeled secondary antibodies and other second step reagents, labeled receptor ligands, and antibodies with species cross-reactivity.

Conclusions: Using refined technical approaches, the number of parameters analyzed by flow cytometry per cell sample can be greatly increased, enabling multidimensional analysis of rare samples and giving critical insight into veterinary and other less commonly analyzed species. By maximizing information from each cell sample, multicolor flow cytometry can reduce the required number of animals used in a study.

Keywords: Flow cytometry, Multiparameter, Veterinary species

Background

Fluorescence-activated cell sorting (FACS) and flow cytometry have been essential immunological tools since the invention of FACS in the late 1960s [1–3], as they enable identification, characterization, and isolation of defined leukocyte subsets [4, 5]. Flow cytometry employs fluorochrome-labeled antibodies that detect cell surface or intracellular antigens [6, 7], a method that was first developed for characterization of cells and tissues by microscopy [8]. Recent advances in the development of novel fluorochromes and instrumentation (i.e. flow cytometry analyzers and sorters) allow for the theoretical analysis of up to 50 parameters in a single staining panel [9], and a 28-color panel has recently been demonstrated [10]. Polychromatic experiments enable the simultaneous measurement of a larger number of cell surface and intracellular markers, thereby facilitating the analysis

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of infrequent cell subsets or limited cell samples [5, 11–13]. Therefore, many institutions have acquired high capacity flow cytometers, and the analysis of > 10 fluorochromes has become routine in the study of human and mouse cells.

The house mouse (*Mus musculus*) is the most frequently used species in biomedical research and, as a consequence, a large spectrum of reagents and genetic models are available [14, 15].. However, animal species other than the house mouse may represent more suitable models of specific human physiology, disease or anatomy, and can also enable studies of comparative medicine and/or of zoonotic pathogens in their natural hosts [14, 16, 17]. An example is the guinea pig, which has been a model for human infectious diseases for 200 years, and enabled disease research and vaccine development in tuberculosis [18, 19]. More recent examples for the use of non-mouse species in biomedical research include pigs and sheep in orthopedics and Alzheimer's disease [20–22] and dogs in oncology [23].

Flow cytometry is a key method in immunological studies [24] encompassing biomedical, veterinary, agricultural, and wildlife research, but the method is also routinely employed in veterinary clinical laboratories [25, 26]. Unfortunately, we face many limitations in the analysis of non-mouse animal samples, including lower availability of commercially or otherwise available antibodies to cell antigens and reduced options for fluorochrome labels by commercial antibody suppliers. It is also not uncommon to receive limited amounts of hybridoma supernatant rather than purified antibody. In addition, antibodies for non-standard species tend to be more expensive. Due to this absolute and relative lack of reagents, the design of state-of-the-art multicolor flow cytometry staining panels is much more difficult than it is for mouse or human cell samples.

Our laboratory studies lymphocyte recirculation using lymph vessel cannulation in sheep, which was pioneered by Bede Morris [27, 28]. Due to a number of limitations, afferent lymph vessels cannot be readily cannulated in mice or humans, and lymph vessel cannulation in sheep allows for the analysis of lymphocytes during their physiological recirculation through tissues [29–31]. Here we present technical approaches that are commonly employed by our laboratory to increase the number of parameters analyzed by flow cytometry per cell sample from sheep [32–36]. The discussed approaches are compatible with the analysis of cells from most other animal species and include direct modification of antibodies by covalent conjugation or Fc-directed labeling (Zenon™ labeling kits), labeled secondary antibodies and other second step reagents, labeled receptor ligands and species cross-reactivity. Detailed guidelines for the use of flow cytometry, including general protocols, are extensively discussed elsewhere [11, 24].

Results

Selection of fluorochromes

When designing multicolor staining panels for flow cytometry, one is limited to the use of fluorochromes compatible with available flow cytometers. Therefore, the technical specificities of the available instrumentation determine the fluorochromes in a staining panel. For the panels presented in this paper we used the BD LSR Fortessa™ cell analyzer. Our machine has 5 lasers, UV (355 nm), violet (405 nm), blue (488 nm), yellow/green (561 nm), and red (640 nm), and can simultaneously detect up to 18 colors plus forward- (FSC) and side scatter (SSC) properties (Fig. 1). Figure 1 depicts the specific laser and filter set-up of our flow cytometer, its theoretically available colors, as well as examples for fluorochromes commonly used in our laboratory. As advised [37], we aim to choose fluorochromes with minimal spectral overlap, and online resources such as the Spectrum Viewer from BD, Fluorescence Spectra Viewer from Thermo Fisher Scientific, or the BioLegend Spectra Analyzer help with assessing the degree of spectral overlap and potential spillover. The simultaneous use of fluorochromes with extensive spectral overlap is more feasible with appropriate compensation, carefully titrated antibodies, and when the antibodies recognize distinct cell populations, e.g. B cells vs. T cells; the approach is less suitable for co-expression studies [24, 38]. However, each specific staining panel will need to be tested and optimized on available instrumentation. More details on

Instrument: BD LSR Fortessa™																				
Laser	UV Laser (355 nm)		Violet Laser (405 nm)							Blue Laser (488 nm)			Yellow/Green Laser (561 nm)					Red Laser (640 nm)		
Filter	379/28	450/50 410LP	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	695/40 690 LP	586/15	610/20 595LP	670/30 635LP	710/50 690LP	780/60 750LP	670/14	730/45 690LP	780/60 750LP
Fluoro-chrome examples	•BUV395	•DAPI •AF350	•BV421 •Pacific Blue •Cascade Blue •AF405 •V450	•BV510 •Pacific-Orange	•BV605 •Q-dot-655	•BV650	•BV711	•BV785		•SSC	•FITC •AF488 •CF488 •GFP	•PerCP-Cy5.5 •PerCP •PerCP-eF710	•PE	•AF594 •PE-CF594 •PE-Texas-Red •PE-Dazzle 594	•PE-Cy5	•PE-Cy5.5	•PE-Cy7 •APCeF780	•APC •AF647	•AF700 •APC-R700	•APC-Cy7 •APC/Fire750 •APC-H7 •APC-eF780

Fig. 1 Flow cytometry laser and fluorochrome chart used for our studies. The BD LSR Fortessa™ used in this study is depicted with our laser and corresponding filter set-up, as well as fluorochrome examples commonly used in our laboratory. Abbreviations: AF™, Alexa Fluor™; APC, allophycocyanin; BUV, Brilliant UltraViolet™; BV, Brilliant Violet™; CF, Cyanine-based fluorescent dye; Cy, Cyanine; DAPI, 4′,6-diamidino-2-phenylindole; eF, eFluor; FITC: fluorescein isothiocyanate; LP, long pass; PE, R-phycoerythrin; PE-Cy5, phycoerythrin-cyanine5 conjugate; PE-Cy7, phycoerythrin-cyanine7 conjugate; PerCP, peridinin chlorophyll-A protein

optimal fluorochrome combinations and discussions on appropriate compensation techniques are described elsewhere [12, 24, 37].

When designing staining panels, we select brighter fluorochromes for antibodies that bind rare antigens as advised [37]. Companies such as BioLegend provide a relative brightness index for fluorochromes but also warn that the brightness can vary depending on the antibody, antigen, or cell type, and that it is also influenced by instrumentation. Consequently, titrating the antibody is always recommended. Examples of brighter fluorochromes that we used in our panels of this paper include phycoerythrin (PE), Alexa Fluor™ (AF™) 594, phycoerythrin-cyanine 7 (PE-Cy7), allophycocyanin (APC), and Brilliant Violet™ 421 (BV421) (Fig. 1). In an ideal scenario, monoclonal antibodies for each cell antigen are available in all possible fluorochromes. However, even for human antigens this is not the case and is further from reality in veterinary species. Thus, initial antibody staining panel design will depend on easily available and previously validated antibodies ("what is in the refrigerator") and approaches to expand the panel.

Covalent labeling of antibodies and species-cross-reactive antibodies

Antibody vendors have a variable supply, and antibodies for veterinary species are generally available in a limited number of fluorochrome labels and are often unconjugated. BioRad, for example, has a sound variety of anti-ovine and other veterinary antibodies, most of which are only available purified or conjugated to fluorescein isothiocyanate (FITC) or PE. A method to broaden the fluorochrome range is to label purified antibodies by using a reactive labeling kit that covalently binds fluorochromes to reactive protein groups, such as amines. In contrast to purified antibodies, non-purified antibodies (i.e. hybridoma supernatants, ascites fluid) cannot be labeled covalently without also labeling other protein components in the fluid, but they can be selectively labeled with the Zenon™ labeling method (discussed below). Covalent labeling kits for variable amounts of antibody are available for numerous fluorochromes from commercial vendors, such as Invitrogen™ by Thermo Fisher Scientific, Novus Biologicals, Abcam, or Biotium™. The labeling procedure follows simple protocols provided by the manufacturer and takes less than 3 hours. The newly labeled antibody is immediately ready for staining but should be titrated and validated prior to use in an experiment. The reactive label works for all animal species and IgG subclasses, and the covalently labeled antibody is stable and can be stored for usage over several months. For example, we labeled purified anti-ovine CD8 with a Pacific Blue™ antibody labeling kit and included it in a multicolor panel to detect $CD8^+$ T cells among sheep blood lymphocytes (Fig. 2a). Within the same staining panel, lymphocytes were additionally gated for γδ T cells (Population A), CD4 T cells (C), B cells (D), and $CD11c^+$ antigen presenting cells (E) distinguished within the high side scatter granulocyte population (Fig. 2a).

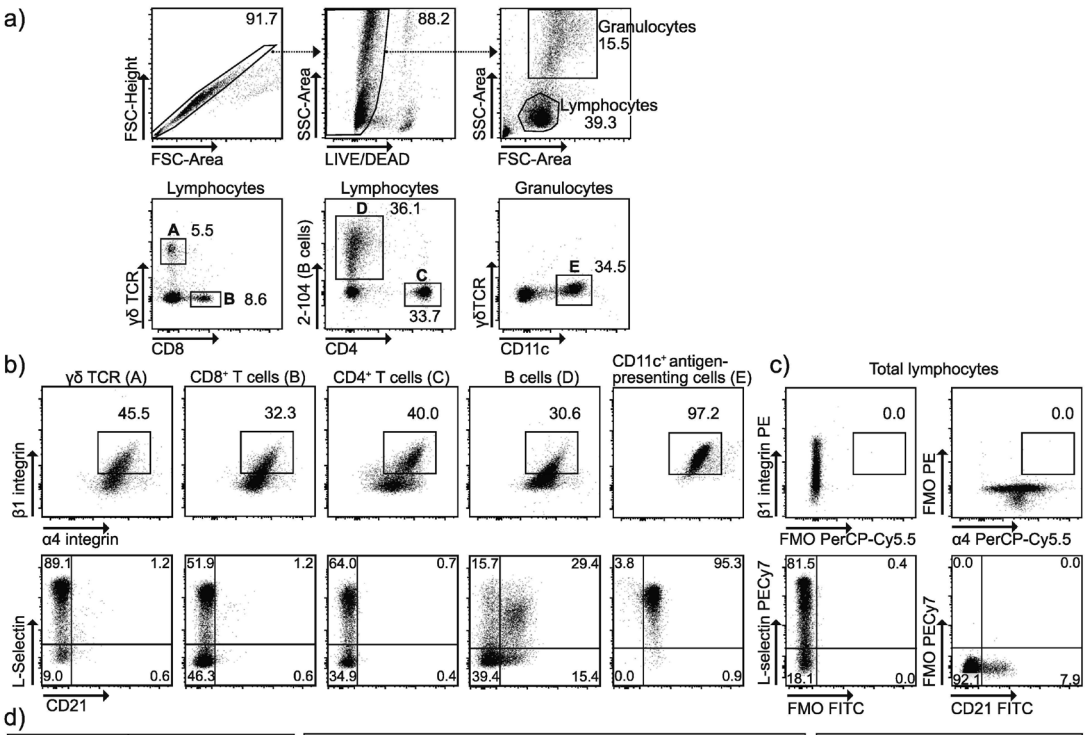
To broaden the antibody repertoire and fluorochrome spectrum, many laboratories use antibodies that are raised against antigens in one species but exhibit documented cross-reactivity for a different species. When antibodies are produced for use in mice and humans, they are more likely to be commercially available in a larger variety of colors. In our example panel, we took advantage of documented cross-reactivity of anti-human α4- and β1-integrin and anti-bovine CD21 antibodies with their corresponding sheep molecules [39, 40] and analyzed all cell subsets (A-E) for these markers, as well as CD62L (Fig. 2b).

Staining controls are particularly important for multiparameter analyses [37]. In a fluorescence minus one (FMO) control all antibodies of the panel, except for one, are included in their respective fluorochromes, allowing assessment of spectral overlap into the "empty" channel (Fig. 2c). Figure 2d depicts the individual fluorochromes and antibodies to antigens used in the staining panel of Fig. 2 and lists the method by which the individual staining was achieved.

Zenon™ labeling kits

An additional approach to overcome limited fluorochrome availability for domesticated and other animal species is the use of Zenon™ labeling kits (Invitrogen™, Thermo Fisher Scientific) [33, 34, 41, 42]. The Zenon™ labeling technique uses fluorochrome-labeled Fab antibody fragments that recognize the IgG subclass (Fc portion) of its target antibody (Fig. 3). This noncovalent conjunction enables the labeling of human, mouse and rabbit antibodies, which can be purified antibodies, hybridoma culture supernatant, or ascites fluid [43]. Therefore, the Fab fragment of the Zenon™ kit has to recognize the species (human, mouse or rabbit) and the IgG subclass (IgG_1, IgG_{2a} or IgG_{2b}) of the specific target antibody. To label the target antibody, it is mixed with the fluorochrome-labeled Fab fragments of the Zenon™ kit, and the mixture is incubated for 5 min (Fig. 3a). Next, the Zenon™ blocking reagent is added to the mixture and incubated for another 5 min (Fig. 3b). The Zenon™ blocking reagent is a nonspecific immunoglobulin mix from the same species as the target antibody and will bind to excess fluorochrome-labeled Fab fragments (Fig. 3b). Finally, together with other antibodies of the staining panel, the Zenon™ mixture is added to the cell sample for staining (Fig. 3c). After washing to remove unbound nonspecific immunoglobulins, the cells are ready to be analyzed by flow cytometry (Fig. 3e).

For the labeling of purified monoclonal antibodies, our laboratory follows the Zenon™ labeling protocol provided



d)

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 strepavidin					Labeled secondary mAb			

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Staining and gating strategy to identify leukocyte populations in ovine blood. Peripheral blood mononuclear cells were obtained by density gradient centrifugation and (**a**) cells were gated on FSC-Height and FSC-Area to exclude doublets. Singlets were further gated on SSC-Area and LIVE/DEAD™-dye^{low} cells to exclude dead cells. Viable cells were then gated for lymphocytes and granulocytes based on FSC and SSC properties. Lymphocytes were gated for γδ T cells (**a**), CD8$^+$ T cells (**b**), CD4$^+$ T cells (**c**), and B cells (**d**). Granulocytes were gated for CD11c$^+$ antigen presenting cells (E). (**b**) Each leukocyte subset (A-E) was analyzed for expression of α4- and β1-integrins (top row) and CD21 and CD62L (bottom). (**c**) Fluorescence-minus-one controls (FMO) for fluorochromes used to stain α4- and β1-integrins, CD21, and L-selectin in (**b**) using the total lymphocyte gate shown in Panel (**a**). (**d**) Table indicating the reagents and flow cytometer configuration used in this staining panel, including the respective fluorochrome, as well as the methods employed to visualize each ovine cell surface marker. Reagents that were obtained as covalently labeled reagents are marked as "directly conjugated". (a and b) Numbers within dot plots represent percentages. Abbreviations: AF™, Alexa Fluor™; Bio, biotin; BV, Brilliant Violet™; FITC, fluorescein isothiocyanate; LP, long pass; mAb, Monoclonal antibody; PE, R-phycoerythrin; PE-Cy7, phycoerythrin-cyanine7 conjugate; PerCP-Cy5.5, peridinin chlorophyll-A protein-cyanine; SA, streptavidin

with the kit, which recommends the use of 1 μg of target antibody, 5 μl of the Zenon™ labeling complex and 5 μl of the Zenon™ block. For some very bright fluorochromes and antibodies that recognize highly expressed cell surface antigens, such as anti-γδ TCR antibody labeled with Zenon™ AF™594 (Fig. 2), we only use 2.5 μl of the Zenon™ labeling Fab fragment and 2.5 μl of the Zenon™ blocking reagent to label 1 μg of antibody. However, for antibody hybridoma supernatants of unknown concentration we found the use of 10 μl supernatant and 5 μl of each the Zenon™ labeling complex and Zenon™ block works best in most cases and may be determined by titration for each batch of supernatant. Figure 2 shows an example of antibody supernatant labeling to stain CD11c$^+$ antigen presenting cells.

One option that the Zenon™ protocol provides is to stop the labeling after the first step (before adding the blocking reagent) for storage at 4 °C. While our laboratory sometimes stores the Zenon labeled antibody for 1–2 h in the refrigerator, the manufacturer's protocol allows for storage of up to several weeks with the addition of 2 mM sodium azide (Invitrogen™). The use of the Zenon™ labeling kits is quick and allows flexibility in staining because the fluorochrome can easily be changed by using the isotype-specific Zenon™ kit in a different color. Multiple Zenon™ labeled antibodies can be used simultaneously in the same staining panel, and isotype controls can be labeled in the same manner as the staining antibodies. Each new antibody-Zenon combination should be titrated to determine the best dilution.

Labeled secondary antibodies and other second step reagents

For the flow cytometric analysis of cells from veterinary species fluorochrome-labeled secondary antibodies are widely used. Monoclonal and polyclonal secondary antibodies are produced in a diverse array of host species and are commercially available in a broad range of colors. The use of monoclonal secondary (and primary) antibodies is preferred as they usually achieve more consistent staining with less background. Secondary antibodies are employed to bind primary antibodies (which recognize antigens on target cells) without cross-recognition of target species antigens. Therefore, secondary antibodies recognize the species and isotype of the primary antibody and are adsorbed to or otherwise unreactive with antigens on cells of the target species. For example, a monoclonal mouse IgM antibody that recognizes sheep B cells (clone 2–104) [44] was visualized with a BV650-conjugated rat anti-mouse IgM monoclonal antibody (Fig. 2a and d). In the same staining panel, mouse anti-ovine CD62L clone DU1–29 was detected with a PE-Cy7-conjugated rat anti-mouse IgG_1 monoclonal antibody (Fig. 2b and d). Thus, use of secondary antibodies leads to signal amplification and is versatile, allowing for the selection of less commonly used fluorochromes and easy matching to a variety of panels.

As a general rule, it is possible to use multiple secondary antibodies in the same staining panel as long as they recognize different immunoglobulin classes or IgG subclasses (e.g. anti-mouse IgG and anti-mouse IgM as shown in Fig. 2, or anti-mouse IgG_1, and anti-mouse IgG_{2a}). Even when using a single secondary antibody, the isotypes of all antibodies in the staining panel must be considered and the primary antibody that is targeted should be the only one recognized by the secondary antibody. However, including two additional staining steps allows for detection of an unlabeled primary antibody in the presence of additional (labeled) antibodies of the same isotype without cross-recognition by the secondary antibody. The first step includes only the primary antibody, followed by only the isotype-specific secondary antibody. After a requisite blocking step with excess unlabeled antibody of the isotype of the target antibody, additional antibodies of the same isotype can be used in a third staining step. Using this approach our laboratory visualized CD62L expressing cells (Fig. 2) as well as natural killer (NK) cells (Fig. 4a and b) with anti-IgG1 secondary antibodies in the presence of other IgG1 staining antibodies. In Fig. 4, unlabeled EC1.1, a monoclonal mouse IgG_1 that recognizes the ovine natural cytotoxicity receptor NKp46 [45] was visualized by rat anti-mouse IgG_1, clone A85–1, conjugated to BUV395 (Fig. 4a and b). Following the secondary labeling, the cells were blocked with non-specific polyclonal mouse IgG to saturate any free valences of the secondary antibody, rendering it unable to interfere

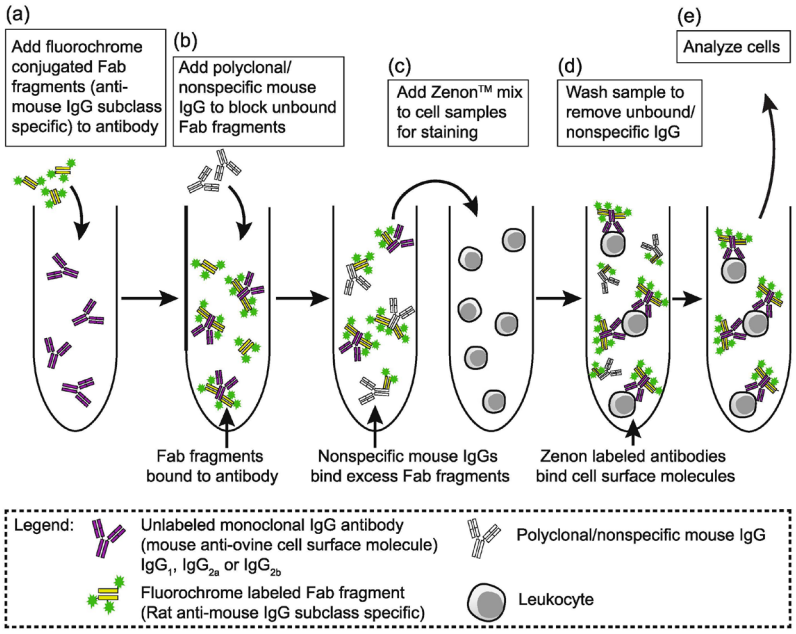


Fig. 3 The principle of Zenon™ antibody labeling. To label mouse monoclonal IgG antibodies, we employed Zenon™ technology. All steps were performed at room temperature. (**a**) The unlabeled target monoclonal mouse IgG (purple) is mixed with mouse IgG subclass-specific fluorochrome-conjugated Fab fragments (green/yellow) from the corresponding Zenon™ kit. The fluorochrome-conjugated Fab fragments bind the target antibody. (**b**) Addition of nonspecific polyclonal mouse immunoglobulin (white) blocks excess unbound Fab fragments. (**c**) The mix of newly Zenon™-labeled antibodies and blocked excess Fab fragments is added to the cell sample and binds to its respective cell surface antigens. Antibodies (Zenon-labeled or directly conjugated) to other cell surface molecules can be included in this step. (**d**) Excess antibody and blocked Fab fragments are washed away, and (**e**) the stained cells are ready to be analyzed or fixed

with the other mouse IgG_1 antibodies in the staining panel. Finally, mouse IgG_1 antibodies against α4- and β1-integrins were employed in the staining procedure (Fig. 4a and b). Isotype control antibodies of the same species, antibody isotype and fluorescent label can be incorporated in the same manner as the staining antibodies (Fig. 4c).

Biotinylated antibodies also increase the fluorochrome spectrum and signal in flow cytometry. Streptavidin is commercially available in many different fluorochromes and binds biotin on the primary antibody with high affinity. This leads to signal amplification, making it particularly useful for detection of antigens with low density per cell, and stressing the importance to titrate both the biotinylated primary antibody and the conjugated streptavidin. Many antibodies are commercially available in biotinylated format and purified antibodies can be biotinylated using antibody/protein biotinylation kits or Zenon™ technology (see above).

Labeled receptor ligands

When antibodies for cell surface receptors are unavailable or when ligand binding ability, as opposed to simple receptor expression is the aim of the study, labeled ligands can be used in flow cytometry. Employing this method, we have previously analyzed ovine lymphocytes for expression of costimulatory molecules B7.1/B7.2 and skin homing marker E-selectin ligand (epitopes that include cutaneous lymphocyte antigen) by evaluating binding of CTLA4-human IgG and mouse E-selectin-human IgG_1 chimeric proteins, respectively [32–34]. Here, we show an example in which E-selectin binding was visualized with an APC-conjugated mouse monoclonal antibody that recognizes the human IgG_1 portion of the E-selectin chimeric protein (Fig. 4d). After gating lymphborne CD4 T cells (as in Fig. 2a), we analyzed their percentage of E-selectin ligand expression (Fig. 4d). Because E-selectin binding requires calcium, a control staining was performed in EDTA-containing buffer (Fig. 4d). Alternative controls will depend on the specific ligand used in an experiment, and examples include staining with irrelevant IgG fusion proteins or blockade of staining with excess unlabeled ligand.

Discussion

In this methods paper, we present several approaches to overcome flow cytometry limitations in the analysis of

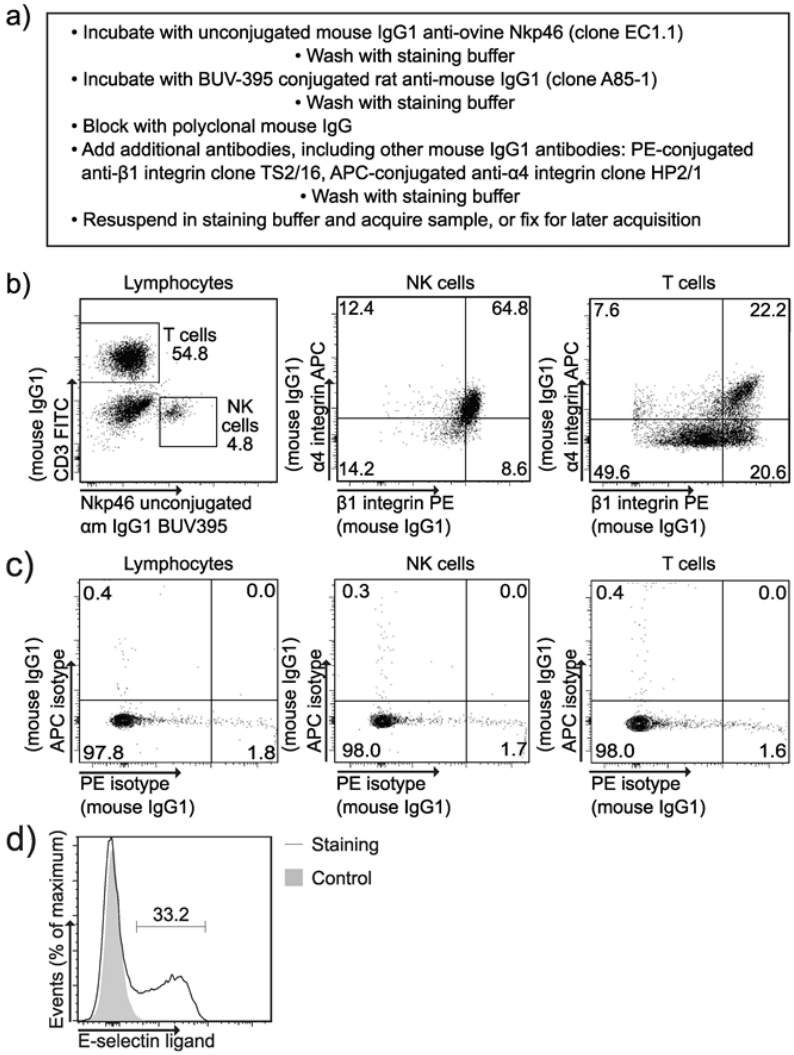


Fig. 4 Secondary antibody staining with multiple same-isotype primary antibodies and cell-surface molecule detection by ligand binding. (**a**) Summary of the steps to stain with an isotype-specific secondary antibody when its target isotype antibody is present multiple times in the same staining panel. (**b**) Staining of NKp46 with an isotype-specific secondary antibody (anti-mouse IgG_1) in the presence of anti α4- and β1-integrin antibodies, which are of the same isotype as the anti NKp46 antibody. Peripheral blood mononuclear cells were pre-gated on single live lymphocytes as in Fig. 2a and $CD3^+$ T cells and $CD3^-NKp46^+$ NK cells analyzed for expression of α4- and β1-integrins. (**c**) Corresponding isotype control staining for α4- and β1-integrins. (**d**) E-selectin ligand expression on $CD4^+$ T cells from afferent lymph of adult sheep was determined by flow cytometry using an E-selectin-human IgG fusion protein. Cells were pre-gated as in Fig. 2a. As a negative control, staining was performed in buffer containing EDTA. (**b**-**d**) One representative of five individually analyzed sheep is shown. Abbreviations: αm, anti-mouse; APC, allophycocyanin; BUV, Brilliant ultra violet; FITC, fluorescein isothiocyanate; PE, R-phycoerythrin

veterinary species. During our studies we faced several technical issues. For example, certain antibody clones were not sufficiently labeled by direct covalent labeling kits. This is an old problem and known causes of labeling resistance include: buffer components react with the dye, suboptimal pH, or reactive amine groups lie within the antigen-binding site of the antibody [46]. Zenon antibodies can be a solution for labeling monoclonal IgG antibodies that do not label well with labeling kits. For example, our ovine CD4 monoclonal antibody (44.38) did not yield satisfying staining quality when conjugated with the Invitrogen Pacific Blue antibody labeling kit. However,

using the Zenon technology yielded superior results. Another common difficulty is the conjugation of IgM antibodies because most labeling kits raise pH and denature the pentameric structure of IgM [47]. Some manufacturers, such as Thermo Fisher Scientific, offer specific protocols optimized for IgM labeling. In the case of the (mouse IgG1) anti-NKp46 clone, neither covalent conjugation nor Zenon™ technology were effective methods for labeling, and we had to employ the staining method outlined in Fig. 4a. Unfortunately, not all commercial antibody suppliers have consistent quality controls in place and we have occasionally seen commercially labeled antibodies that are unreliable. We also found that in one case the Zenon™ mix interfered with the staining of a different antibody in the same staining panel. Specifically, the anti-ovine B cell antibody (2–104) is a mouse IgM and its detection by an anti-mouse IgM secondary antibody was blunted. An ELISA revealed that the Zenon™ blocking reagent that is included in the kits contained both mouse IgG and mouse IgM, and the latter was competing with our mouse IgM primary antibody for binding by the secondary anti-mouse IgM antibody. We solved the issue by using purified IgG for blocking rather than the Zenon™ kit blocking component.

Certain tandem dyes are sensitive to degradation, leading to a weaker signal and detection in other fluorochrome channels. For example, PE-tandem dyes are susceptible to degradation by handling, storage, and light [48, 49]. We also found that the tandem dye PE-Cy7, is sensitive to extended fixation with PFA, which can degrade the fluorophore and lead to artifactual strong signals in the PE channel. We found that the following precautions prevent tandem conjugate degradation: staining at 4 °C in the dark, careful and extensive washing after fixation (i.e. twice with sufficient buffer volume), and storage at 4 °C in the dark for no longer than 24 h. Another potential issue, which we have not encountered in our studies, is the interference of Brilliant Violet and other ultra-bright antibodies with each other when used in the same panel. Such issues can be addressed by using specialized staining buffers. BD Bioscience, for example, offers a specific buffer for staining with Brilliant™ dyes [50].

While we present several simple approaches to broaden the number of flow cytometric parameters per cells, additional approaches exist that we have not utilized so far. For example, PrimeFlow™ (Invitrogen™) or Branched DNA method is a technique to detect cell-expressed RNA by flow cytometry [51], and custom antibody production or customized antibody labeling are also available.

Conclusion

Here, we presented multiple relatively simple and time-tested approaches that broaden the fluorochrome spectrum for flow cytometric analysis of cells from veterinary relevant species. These approaches can enhance the quality and quantity of information obtained from each cell sample. Therefore, the use of multiparameter analysis in flow cytometry can give critical insight into veterinary and other less commonly analyzed species, can help obtain information from rare cell samples, better define subpopulation of cells, and also potentially reduce the required number of animals used in a study.

Methods

Animals and lymphatic cannulation

Eight–eighteen months old female or wether Dorset or Dorset-cross sheep with negative Q-fever serology were purchased from Archer Farms, Inc. and conventionally housed in standard pens, under a 12-h-light/dark cycle, in groups and singly when entering experiments. Hay and water were provided ad libitum, and standard pellet feed for ruminants (Labiana) were fed twice per day. Sheep were 40–65 kg of weight when entering experiments Lymphadenectomy to remove the subiliac (prefemoral) lymph nodes was performed as previously described [52]. Six–eight weeks after lymphadenectomy, pseudoafferent lymph vessels were cannulated with heparin-primed 3 or 3.5 French polyurethane catheters (Access Technologies) in a surgical procedure as described [52]. Pre-procedural sedation was induced with Tilzolan (tiletamine and zolazepam; Dechra) at 4–6 mg/kg into muscles of the hind or front leg; anesthesia was induced with propofol i.v. at 2–8 mg/kg (PropoFlo 28, Zoetis) and/or sevoflurane (Patterson Veterinary) per inhalation at 2–3% in oxygen via mask, and anesthesia was maintained at a surgical plane with 2–3% isoflurane (Isothesia, Covetrus) in oxygen, administered via an endotracheal tube. All surgical procedures were performed under aseptic conditions in a dedicated surgical suite. Postoperative analgesia was provided using buprenorphine (Par Pharmaceuticals) at 0.01–0.05 mg/kg every 4–12 h s.c. in the neck, and/or flunixin meglumine (Flunixin Injection, Norbrook) at 1 mg/kg every 8–24 h i.m. in the leg. Additional doses of analgesics were given if animals showed signs of pain or distress, which were assessed at least three times per day for 3 days, and at least every 12–24 h thereafter for a week. Afferent lymph was collected into sterile bottles containing 100 μL of 10,000 U/mL Heparin (Hospira, Inc.). Collection bottles were changed every 4–12 h. After conclusion of experiments, the animals were euthanized while under anesthesia by i.v. injection with pentobarbital and phenytoin (SomnaSol, Covetrus) at 97.5–195 mg/kg and 12.5–25/kg, respectively. Death was confirmed by auscultation for cardiac arrest. The method of euthanasia is consistent with the recommendations by the Panel of Euthanasia of the American Veterinary Medical Association.

Cell isolation and blood collection

Blood was collected from the jugular vein with a syringe containing heparin. Mononuclear cells were isolated using density gradient centrifugation with Histopaque®-1083 (Sigma-Aldrich). Blood was diluted at a one to one ratio with elution media (58.4 mM sucrose (Sigma-Aldrich), 10 ml 5 mM EDTA (Invitrogen), 100 mL 10x PBS (Gibco), 900 mL Milli-Q Water) at room temperature and carefully layered on top of the Histopague in a conical tube. The layered blood is centrifuged at 9000 RCF for 30 min. Lymphocytes are collected by harvesting the buffy coat. Blood and lymph-borne cells were washed with wash media (RPMI 1640 medium with GlutaMAX™ (Gibco®), 0.2% BSA (Sigma-Aldrich), and 25 mM HEPES (Gibco®)), and, when necessary, red blood cells were lysed using red blood cell lysing buffer (155 mM ammonium chloride (Sigma-Aldrich), 10 mM sodium bicarbonate (Sigma-Aldrich), and 0.1 mM EDTA (Gibco)). Isolated cells were resuspended in wash media, counted by hemocytometer, and kept on ice until staining.

Flow cytometry

Mouse monoclonal anti-ovine antibodies recognizing the following ovine antigens were used in the study: CD4 (clone 44.38; BioRad), CD8 (38.65; BioRad), γδ TCR (86D; Washington State University Monoclonal Antibody Center), CD62L (DU1–29; Washington State University Monoclonal Antibody Center), and NKp46 (EC1.1; Timothy Connelly and Lindert Benedictus, The Roslin Institute); CD11c (17–196) and pan-B cell marker (2–104) were kindly provided as hybridoma supernatant or purified antibody by Alan Young (South Dakota State University) and Isabelle Schwartz-Cornil (Institut national de la recherche agronomique). The following mouse monoclonal antibodies, cross-reactive with sheep antigens [39, 40], were used: α4-integrin (HP2/1; Novus Biologicals), β1-integrin (TS2/16; eBioscience), and CD21 (CC21; BioRad). The following secondary antibodies were employed in our study: rat anti-mouse IgG_1 BUV395 (A85–1, BD Biosciences), rat anti-mouse IgG_1 PE-Cy7 (RMG1–1; Biolegend), rat anti-mouse IgM (II/41, BD Biosciences), and mouse anti-human IgG_1 APC (97,924; R&D Systems). Streptavidin-conjugated PerCP-Cy5.5 (BD Bioscience) was used as a secondary or tertiary step reagent. Unlabeled antibodies were labeled with Zenon™ kits or Molecular Probes (Invitrogen™) antibody/protein conjugation kits, as indicated in each Figure, following the manufacturer's instructions (Invitrogen™, ThermoFisher Scientific). The Zenon™ labeling blocking step was performed with purified whole mouse IgG (Jackson ImmunoResearch).

All staining steps were performed in a total volume of 100 μl on ice. Cells were washed with staining buffer (Dulbecco's phosphate-buffered saline (DPBS; Corning) and 0.2% tissue-culture grade bovine serum albumin (BSA; Sigma-Aldrich)) and spun down at 500 RCF. 2×10^6 cells were stained per 1.2 mL microtiter tube (Fisher Scientific). To block nonspecific binding, each tube of cells was resuspended, and subsequently incubated for 10 min, in 10 μl of staining buffer containing 1 μg sheep IgG (Jackson ImmunoResearch) and/or 1 μg mouse IgG (Jackson ImmunoResearch), as well as 0.1 μl of the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen™). The blocking step was performed at the beginning of the staining process or after staining with a secondary antibody before addition of antibodies of the same isotype as the primary antibody (Fig. 2 and Fig. 4 a and b). After blocking, antibodies to cell surface antigens were added and cells incubated for 15 min, and subsequently washed with staining buffer. Cells were then fixed by resuspending and incubating for 15 min in 2% paraformaldehyde (Sigma Aldrich), followed by washing with staining buffer. After fixation, ovine CD3 staining was performed in staining buffer containing 0.5% saponin (from *Quillaja* bark, molecular biology grade; Sigma Aldrich). Binding to a mouse E-selectin-human IgG_1 chimeric protein (R&D Systems) was tested in DPBS containing calcium and magnesium (Corning), and visualized by an APC-conjugated mouse anti-human IgG_1 antibody (clone 97,924; R&D Systems). The control was stained in the same manner using DPBS without calcium and magnesium under the addition of 30 mM ethylenediamine tetraacetic acid (EDTA; Invitrogen™). Data was acquired using the BD LSR Fortessa™ (BD Biosciences) and analyzed with FlowJo software (Tree Star). A single cell gate was set for each cell sample using FSC-Area and FSC-Height as depicted in Fig. 2a. Dead cells were excluded from analysis by gating on LIVE/DEAD™ Fixable $Aqua^{low}$ events, and lymphocyte and/or granulocyte gates were drawn based on SSC-Area and FSC-Area (Fig. 2a). A minimum of 100,000 lymphocytes were recorded per tube.

Abbreviations

AF™: Alexa Fluor™; APC: Allophycocyanin; BSA: Bovine serum albumin; BV: Brilliant Violet; DPBS: Dulbecco's phosphate-buffered saline; EDTA: Ethylenediamine tetraacetic acid; FACS: Fluorescence activated cell sorting; Ig: Immunoglobulin; MAb: Monoclonal antibody;, NK cells: Natural killer cells; PBMC: Peripheral blood mononuclear cell; PE: Phycoerythrin; PE-Cy: Phycoerythrin-cyanine; PFA: Paraformaldehyde; RBC: Red blood cell; RCF: Relative centrifugal force; RPMI: Roswell Park Memorial Institute

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Authors' contributions

Conceptualization: GFD; Formal Analysis: JH, JTR, GFD; Funding Acquisition: GFD, Methodology: JH, JTR, GFD; Resources: GFD; Supervision: GFD; Validation: JH, JTR, GFD; Visualization: JH, JTR, GFD; Writing - Original Draft

Preparation: JH, GFD; Writing - Review and Editing: JH, JTR, GFD. All authors have read and approved the manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval
All animal procedures were approved under protocol number 01934 by the Institutional Animal Care and Use Committee of Thomas Jefferson University and conducted in accordance with national and institutional guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Publisher's Note

5 Discussion

Flow cytometry is most widely used in immunology [38], and the methods we discuss in *Hunka, J. et al. 2020* were established in immunological studies though can also be replicated outside of this area. Since sheep have been used as animal models for decades, there are more reagents available for sheep than for many other veterinary animal species, and so they represented a natural starting point when discussing how to expand our repertoire of techniques and methods. The publications, which is part of this work, demonstrates how a combination of techniques provide the ability to analyze at least 12 parameters in non-traditional animal species on par with advanced studies performed in rodents and humans. These techniques can be useful for the analysis of cells from most veterinary species and can be employed in research laboratories and in veterinary clinical medicine.

These approaches include the direct labeling of monoclonal antibodies with a desired fluorochrome by covalent conjugation using commercially available antibody labeling kits. Some vendors offer a variety of anti-ovine and other veterinary antibodies, most of which are only available purified or conjugated to a few colors like FITC or R-phycoerythrin (PE). Antibody labeling kits are easy to use and can be employed for all animal species and IgG subclasses. Furthermore, once labeled, antibodies can be stored for several months. This makes antibody labeling kits a good method for veterinary clinicians and researchers who want to expand their choices of colors for flow cytometry. It can also be profitable in veterinary clinical medicine for diagnostics. As described in the fundamentals, flow cytometry can be used to classify types of hematolymphoid neoplasms, like lymphoma. Staining for certain antigens, like CD3, CD4, CD1, CD21 and MHCII is an integral part in this classification [49]. With the help of antibody labeling kits, a proven antibody-fluorochrome combination can be labeled in larger quantities, stored and reused for diagnostic purposes. A quicker, short term method of labeling antibodies in a desired color is the Zenon™ technology, a Fc-directed, noncovalent conjunction of fluorochromes to antibodies. It allows for the labeling of antibodies raised in human, mouse and rabbits, which can be purified antibodies, hybridoma culture supernatant, or ascites fluid. It is quick and allows flexibility in staining because the fluorochrome can easily be changed by using the isotype-specific

Zenon™ kit in a different color. However, it cannot be used for all species and is therefore less versatile in its applications.

In my work with sheep I regularly employed second step reagents, like labeled secondary antibodies. They are extensively used with a wide array of host species available and they are also sold in less commonly used fluorochromes. They have proven useful when supernatants or hybridoma antibodies are employed, which happens regularly with veterinary species where antibody availability is particularly limited. Covalent antibody labeling kits do not work with supernatants and Zenon™ kits are expensive and not available in less commonly used fluorochromes like PECy7 or BV421. Second step reagents can easily be matched to a variety of panels and can help to enable multicolor flow cytometry analysis in more species. In addition, this work demonstrates a popular fall back when using alternative species: cross-reactive antibodies. As described above, various human and other antibodies have been tested for species cross reactivity. It is regularly employed in veterinary species. These antibodies have proven themselves very useful in the investigation of the immune system in veterinary medicine, especially ruminants. [68]. Lastly, we utilized labeled receptor ligands that can be employed to further enhance a reagent repertoire when antibodies for cell surface receptors are unavailable or if the aim of the study is to specifically investigate ligand binding abilities. The use of receptor ligands can give a deeper understanding about functional cell adhesion and cell trafficking throughout the body to target cells in inflammation and mediate immune responses.
Overall, utilizing the power of multiparameter flow cytometry to investigate the immune system of different species can improve animal and human lives by making it possible to control and treat diseases more effectively and safely [18]. This is crucial for developed and developing countries, as diseases like classical swine fever, paratuberculosis, rabies or brucellosis are a risk for domesticated and wildlife species and can threaten human health if they are zoonotic diseases[16].

Comparative immunological studies provide evidence that the immune systems of various animal species are similar but not identical [65, 87-89]. Which is not surprising as the immune system has evolved to protect the host against diseases which occur in a specific environment. Therefore, individual species encounter different threats to their heath and will rely on different elements of the immune

system to protect them [19]. Although most fundamental aspects of the bodies defense systems are evolutionarily conserved across species, it is also important to conduct studies in the species itself to identify unique immune mechanisms. Importantly, using the most appropriate species for a specific research purpose will also reduce the total numbers of animals used in studies [4]. There is an ongoing discussion over the appropriate use of experimental animal models, and it should be a priority to consider the relevance of a certain species for the translation of the research into useful information for other species [5, 16, 90]. As rodents have been the primarily utilized species [4] the limitations that come with less popular species can be challenging and therefore, make it hard to use multiparameter analysis and their advantages [66]. However, there are a lot of initiatives for the improvement of immunological knowledge in veterinary species[16]. There are multiple consortia and committees, like the Veterinary Immunology Committee (VIC). The VIC established the VIC Toolkit, a consortium with the mission "to provide a global network for veterinary reagent availability and facilitate information exchange". [19] This organization plans workshops for reagent development, commercialization and provision to the veterinary immunology research community [18]. As part of this effort "The Immunological Toolbox" was developed. An online resource that helps finding location and supply for reagents and associated methods for veterinary immunological research [91]. Flow cytometry is evolving and more commercial antibodies are becoming available [12] and cross reactivities continue to be discovered, which will open up possibilities for veterinary medicine in the future. In the meantime, utilization of the techniques described in this thesis work will immediately expand the ability of veterinarians and other researchers to thoroughly investigate a multitude of research questions and disease states in veterinary species This can offer insight into immune system of veterinary species where a broader knowledge is crucial for the diagnosis, prevention and treatment of disease and overall animal welfare and health [19].

6 Summary

In recent years is has become difficult to imagine immunological studies without flow cytometry. There is an extensive amount of flow cytometers, reagents and resources available for those who wish to examine the immune system through flow cytometric studies. Through multiparameter analysis it is possible to obtain important information about leukocyte, or other cell populations, in an individual or a species, to establish a broader knowledge about the immune system and its mechanisms. Unfortunately flow cytometric analysis in veterinary species come with obstacles and limitations, such as the lack of commercially available antibodies or limited color choices. This makes it hard to establish state-of-the-art analysis in these species.

This work aimed to demonstrate the importance of the use of flow cytometry in immunological studies in veterinary clinical medicine and research and presents refined technical approaches to increase the number of parameters analyzed by flow cytometry per cell sample. These approaches include the direct labeling of monoclonal antibodies with a desired fluorochrome by covalent conjugation using commercially available antibody labeling kits, or for immediate use and increased flexibility using Fc-directed Zenon labeling technology; second step reagents like labeled secondary antibodies are also discussed. In addition, this work demonstrates a popular fall back when using alternative species: cross-reactive antibodies. Lastly, we discussed labeled receptor ligands that can be employed to further enhance a reagent repertoire. These techniques provide the ability to analyze at least 12 parameters in non-traditional animal species on par with advanced studies performed in rodents and humans. This can give insight into immune system of veterinary species where a broader knowledge is crucial for the diagnosis, prevention and treatment of disease and overall animal welfare and health. [19]

7 Zusammenfassung

Heutzutage ist es schwer, sich immunologische Studien ohne Durchflusszytometrie vorzustellen. Wissenschaftlern steht eine große Auswahl an Geräten, Reagenzien und Hilfsmitteln zur Verfügung, um das Immunsystem mit Hilfe der Durchflusszytometrie zu erforschen. Multiparameter-Durchflusszytometrie ermöglicht die Erhebung wichtiger Informationen über Leukozyten, oder andere Zellpopulationen, in einem Individuum oder einer gesamten Tierart. Dies trägt zu einer Erweiterung des Wissens um das Immunsystem und dessen Funktionsweise bei. Leider ist die Analyse von Zellen mithilfe von Durchflusszytometrie in der Veterinärmedizin mit Schwierigkeiten verbunden. Durch Einschränkungen wie zum Beispiel einem limitierten Angebot an monoklonalen Antikörpern oder einer geringen Auswahl an Fluorochromen, kann das volle Potential von Durchflusszytometern in der Veterinärmedizin bislang nicht ausgeschöpft werden.

Das Ziel dieser Arbeit war, die Bedeutung der Durchflusszytometrie für immunologische Studien in der Veterinärmedizin zu verdeutlichen und verfeinerte Methoden zu demonstrieren, durch welche die Nutzung der Multiparameter-Durchflusszytometrie vereinfachen werden kann. Diese Methoden umfassen die Kopplung von Fluorenzfarbstoffen an ungefärbte Antikörper, mittels kovalenter Bindung durch kommerziell verfügbare „Antibody Labeling Kits“ oder mittels kurzfristiger Bindung durch auf Fc-Fragmente gerichtete „Zenon™ Kits“. Darüber hinaus wurde die Nutzung von zweistufigen Reagenzien wie zum Beispiel Farbstoff-markierten Sekundärantikörpern und die häufig verwende Methode der Nutzung von Spezies übergreifenden Antikörpern demonstriert. Zuletzt wurde dargestellt, wie auch Fluoreszenz-markierte Rezeptor-Liganden zu einer Erweiterung des Repertoires an Reagenzien beitragen können. Zusammenfassend konnte die Arbeit demonstrieren, dass mit Hilfe der Anwendung dieser Methoden mindestens 12 Parameter in nicht-traditionellen Tierarten analysiert werden können, welche gleichwertig zu Studien im Mausmodell in der Humanmedizin sind. Dies kann kritische Einblicke in das Immunsystem von veterinärmedizinisch relevanten Tierarten geben, welche kritisch für die Diagnose, Prävention und Behandlung von Krankheiten sind.

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