Flow Cytometric Identification and Enumeration of Monocyte Subsets in Bovine and Water Buffalo Peripheral Blood

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Monocytes are innate immune system key players with pivotal roles during infection and inflammation. They migrate into tissues and differentiate into myeloid effect cells (macrophages, dendritic cells) which orchestrate inflammatory processes and are interfaces between the innate and adaptive immune responses. Their clinical relevance to health and disease of cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*), two of the most important livestock species, has been highlighted in physiologic (pregnancy) and pathologic (mastitis, metritis, and viral infections) conditions.

The existence of three different monocyte subsets in cattle was established by flow cytometry (FC), as follows: classical (cM; $CD14^{++}CD16^{-/low}$), intermediate (intM; $CD14^{++/+}CD16^{+}$), and non-classical (ncM; $CD14^{-/low}CD16^{++}$) monocytes. FC applications for studying the immune system of cattle and water buffalo still have significant limitations. In this article, we describe some practical approaches to overcome these limitations and, in particular, allow the identification and enumeration of cM, intM, and ncM subpopulations in cattle and buffalo peripheral blood. Indeed, we propose the new procedure lyse/wash/no-centrifugation (L/W/NC) that can be combined with the FC absolute counting procedures and can overcome specific issues of the lyse/no-wash protocols (L/NW). Finally, for the first time, we demonstrated the existence of cM, intM, and ncM monocyte subsets also in the water buffalo, showing some interesting differences with cattle, such as the bubaline cM are mainly $CD14^{+/++}/CD16^{+}$. These subtle differences may influence inflammatory disease regulation in, for example, mastitis and metritis. The upregulation of CD16 expression on cM may reveal different monocyte priming, leading to different functional features of macrophages/dendritic cells in tissues after infection. © 2023 Wiley Periodicals LLC.

Basic Protocol: Absolute count of cM, intM, and ncM without compensation
Alternate Protocol: Absolute count of cM, intM, and ncM for single laser platform
Support Protocol 1: In-house monoclonal antibody labeling using a Pacific Blue™ kit

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Support Protocol 2: In-house monoclonal antibody labeling using an Alexa Fluor® 647 kit
Support Protocol 3: Titration of fluorochrome-conjugated antibodies

Keywords: absolute count • *Bubalus bubalis* • cattle • monocyte subsets • single-platform • flow cytometry • bovine peripheral blood • water buffalo peripheral blood

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INTRODUCTION

Monocytes are key players of the innate immune system exploiting crucial functions during infection or injury. As in humans, Hussen and co-workers demonstrated the existence of three different monocyte subsets in cattle (*Bos taurus*). Bovine classical monocytes (cM) showed high levels of CD14 but no CD16 expression ($CD14^{++}CD16^{-}$), while intermediate monocytes (intM) expressed high CD14 and moderate CD16 levels ($CD14^{++}CD16^{+}$). Bovine non-classical monocytes (ncM), instead, showed high CD16 but no or very low CD14 expression ($CD14^{-/low}CD16^{++}$; Hussen et al., 2013). In addition, Pomeroy and collaborators found a significant association between cattle postpartum diseases (mastitis and metritis) and modifications of monocyte subset counts (Pomeroy et al., 2017). Monocytes isolated from cattle and buffalo blood and differentiated into macrophages showed differential phagocytosis and intracellular killing abilities against the bacterial pathogen (*Pasteurella multocida*) with higher efficiency for cattle than buffalo cells (Hasnan, Puspitasari, Othman, Zamri-Saad, & Salleh, 2022).

Absolute enumeration of cell subpopulations by flow cytometry (FC) is a well-established technique with clinical relevance in several human diseases (Brando et al., 2000; Hristov, Schmitz, Nauwelaers, & Weber, 2012; Iannetta et al., 2021). In a dual-platform, absolute cell counts were derived from a flow-cytometric-assessed percent subpopulations within leukocytes, combined with the assessment of the absolute leukocyte count from a hematology cell analyzer. The single-platform method employs fluorescent counting beads and directly generates absolute cell counts from a single flow cytometric assessment (Keeney et al., 1998). As cited, FC using absolute counting bead standards is considered the gold standard for enumerating cell subsets in clinical samples. This procedure is starting to be applied in veterinary medicine, and comparative protocols between human, cattle, and sheep blood are proposed. (Pieper et al., 2016). However, in humans, different protocols for isolating monocytes from whole blood can profoundly affect cell plasticity (Fendl et al., 2019, Zhou et al., 2012), impeding the detection of their activation. Indeed, surface expression markers can change during cell handling (Kiefer et al., 2021), and monocyte subsets can be consequently activated. Our groups previously highlighted the impact and significance of detecting absolute numbers of bovine (Pomeroy et al., 2017) and bubaline (Grandoni et al., 2020) immune cells using the dual-platform approach. Whether different monocyte composition in the two species may have contributed to the functional differences in their overall antimicrobial response still needs investigation. Our setup from whole blood, using single platform, no-centrifugation protocols, represents a significant improvement in obtaining reproducible absolute cell counts, avoiding cell loss and cell function alterations. Through accurate monocyte analyses, the

Table 1 Schematic Representation of Proposed Count Protocols

Protocol/panel	Specificity	Channel[a]	Laser configuration[a]
Basic Protocol/Panel A	CD14	R1 (660/20)	Violet (405 nm)
			Blue (488 nm)
	CD16	B1 (525/40)	Red (638 nm)
	CD172a	V1 (450/45)	
Alternate Protocol/Panel B	CD14	B2 (585/42)	Blue (488 nm)
	CD16	B1 (525/40)	
	CD172a	B3 (690/50)	

[a] Optical configuration of CytoFlex (Beckman Coulter).

setting up of differential monocyte phenotypes represents a potentially powerful and decisive tool in the comprehension of the physiologic and inflammatory immune response.

In this article, we describe practical approaches to allow the identification and absolute count of cM, intM, and ncM subpopulations in cattle and water buffalo peripheral blood. We propose the new procedure "lyse/wash/no-centrifugation" (L/W/NC) to overcome the pitfalls of lyse/no-wash protocols (L/NW; Petriz, Bradford, & Ward, 2018). One basic and one alternative protocol using the single-platform approach will be explained.

- Basic Protocol: A zero compensation protocol using the minimal core panel combination-A, performed on a three-laser flow cytometer (see Table 1, panel A).
- Alternate Protocol: The minimum core panel combination-B, designed for single blue laser instruments (see Table 1, panel B).

Support Protocols 1 and 2 provide detailed instructions for in-house monoclonal antibody (mAb) labeling. Thus, users can overcome current difficulties in the multicolor FC panel design due to the insufficient availability of veterinary labeled mAbs. Finally, Support Protocol 3 helps to titrate labeled mAbs, a crucial step in optimizing L/W/NC protocols.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

ABSOLUTE COUNT OF cM, intM, AND ncM WITHOUT COMPENSATION

BASIC PROTOCOL

This protocol allows the identification and absolute count enumeration of the three monocyte subsets using an L/W/NC procedure on a three-laser flow cytometer without compensating the fluorochrome spillover. It requires only anti-CD14, anti-CD16, and anti-CD172a, as a minimum number (core panel) surface markers to identify monocyte subsets. The single-platform approach allows a one-step absolute subset count reducing laboratory operations and management costs. Fluorescent counting microbeads, and reverse pipetting technique (see Critical Parameters) are needed using this approach. Correctly performed L/NW protocols are accurate, reproducible, rapid, and do not require centrifugation. However, their optimization requires accurate mAbs titration, and optimal temperature and time labeling conditions (see Critical Parameters, Optimizing L/W/NC section).

Protocol pros: Accurate differentiation of intM and ncM, fast and easy execution, low-cost protocol.

Protocol cons: Requires a three-laser instrument and in-house labeling of purified mAbs.

Table 2 List of Monoclonal Antibodies Used for the Proposed Counting Protocols

Specificity	Clone	Labeling	Quantity/concentration[a]	Source	Catalog number	Panel
CD14	MM61A	In-house AF647	60 µg/[∼0.5][b]	WSU-MAC[c]	BOV2109	A
	CC-G33	PE	100 tests/1 ml[d]	Bio-Rad	MCA2678PE	B
CD16	KD1	FITC	100 µg/[∼0.1]	Bio-Rad	MCA5665F	A, B
CD172a	DH59B	In-house PB	10 µg/[∼0.2][b]	WSU-MAC	BOV2049	A
	CC149	PE-Cy5	100 tests/1 ml[d]	Bio-Rad	MCA2041C	B

[a] mAb concentrations are expressed as [µg/µl].
[b] Quantities and concentrations are considered after conjugation reaction (see Support Protocols 1 and 2).
[c] Abbreviation: WSU-MAC, Washington State University-Monoclonal Antibody Center.
[d] Quantities and volumes of commercial labeled mAbs.

CAUTION: All biological specimens, as well as materials coming in contact with them, are considered biohazards; wear suitable protective clothing, eyewear, and gloves according to local regulations and procedures.

Materials

Bovine or buffalo peripheral blood (from the jugular vein, collected into 9-ml anticoagulated tubes, used within 4 hr after sampling; see Understanding Results, Optimizing of 3-color/zero compensation panel)
Multicolor mAb cocktail (Panel A): CD14-AF647/CD16-FITC/CD172a-Pacific Blue (see recipe, and Tables 1 and 2)
Tris-buffered ammonium chloride lyse solution (0.87%, w/v, pH 7.3; see recipe)
Dulbecco's Phosphate Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+} plus 1% BSA (see recipe)
Flow-Count Fluorospheres (Beckman Coulter, cat. no. 7547053)
Milli-Q grade distilled water

Three calibrated single-channel pipets (1-10 µl; 10-100 µl; 100-1000 µl) with sterile disposable tips (preferably with filter)
Polypropylene 75- × 12-mm flow cytometer tubes, preferably with cap (e.g., Sarstedt, cat. no. 55.526.006)
Polypropylene microtube, 0.5 ml
Appropriate tube racks
Biosafety cabinet
Biohazard waste container
Timer
Rotary shaker and vortex mixer (optional)
Benchtop flow cytometer, equipped with three-lasers (CytoFlex Beckman Coulter, Violet-Blue-Red Series, or equivalent)

1. Pipet 50 µl of whole blood at the bottom of the flow cytometer tubes (FC-tube) using the reverse pipetting technique (see Critical Parameters, Accurate and reproducible pipetting section).

   This is an important step for the accuracy and repeatability of the results. Blood specimen must be stored at room temperature until use (maximum 4 hr). The careful mixing of blood in the test tube with a rotary shaker ensures proper specimen homogeneity. Alternatively, invert the test tube at least ten times immediately before use. Quickly dry the external tip surface with laboratory paper. Use all caution shown in Table 3. We recommend simultaneously processing a reduced number of samples (four to six).

2. Pipet 8 µl of the monoclonal cocktail.

   Even using low-retention pipet tips, small amounts of blood remain in the tip. To ensure maximum accuracy, dispense the mAb cocktail onto the internal tube wall and avoid

Table 3 List of Factors Influencing Pipetting Accuracy and Precision

Factor	Actions required
Pre-wetting the tip	The pre-wetting of a new tip should be performed for 2-3 times using reverse pipetting method
Leaky or poorly seated pipet tip	Use original or recommended pipet tips. Fit the pipet tip correctly, avoiding hammering the pipet on the tip rack.
Failure to clean the pipet tip after aspirating the required volume of blood or beads	Wipe the pipet tip with paper starting from the base and avoid touching the tip hole
Pipet tip immersion depth	For dispensing 50 µl, the optimal immersion depth is 2-3 mm
Irregular rhythm and fast pipetting	Pipet slowly (1-2 s for each step) and evenly. Wait 1-2 s at the end of the aspiration and dispensing step.
Malfunctioning of the piston system	Check pipet performance weekly, perform calibration every 6 months, and complete maintenance annually. Use a dedicated pipet only for absolute counting.
Parallax error in volume setting	Place the pipet vertical and perpendicular in front of the eyes then set the required volume
Temperature of ambient, sample, and counting beads	Pipet only when they have reached room temperature

touching the blood. The antigen-antibody reaction is rapid, therefore it is essential to immediately vortex the sample. Alternatively, mix manually with three to four small strokes at the bottom tube.

3. Incubate at room temperature in the dark for 10 min.

   Incubation time must be carefully evaluated during L/W/NC procedure optimization (see Critical Parameters).

4. Add 0.5 ml lyse solution and mix briefly. Place samples in the dark at room temperature for 10 min.

   The lysis time should not be prolonged as it would damage the cells with negative effects on the light-scatter parameters, forward scatter (FSC) and side scatter (SSC). The time required may change, particularly with the animal's age or physiological condition. For example, blood samples from young animals (8-16 months) could require stopping the lysis reaction 3-4 min earlier than for adults.

5. Add 1.0 ml cold DPBS plus 1% BSA. Mix gently and place samples in the dark at room temperature for 10 min.

   The addition of cold DPBS is essential to block the lyse reaction and wash leukocytes.

6. Add 50 µl Flow-Count fluorospheres stirred and pre-warmed to room temperature using the reverse pipetting technique.

   This is the second decisive step for accurate and repeatable results. Counting beads need to be stored at 2°C to 8°C. Use all caution shown in Tables 3 and 4. Ensure that room temperature is reached before dispensing; different temperatures can lead to even significant errors in the pipetted volume. The Flow-Count mixing is crucial for suitable bead resuspension: Mix manually for 15-20 s, wait about 5 s (to avoid air bubble pipetting), dip the tip ∼1 cm below the liquid surface, and aspirate slowly (to prevent air bubble formation). Quickly dry the external tip surface with laboratory paper, pipet the counting beads into the center of the labeled specimen, and vortex briefly. Add counting beads to all samples. Using the reverse pipetting technique, ∼75 µl of Flow-Count are needed for each sample.

   CAUTION: Flow-Count beads contain 1% formaldehyde.

7. Proceed immediately to acquisition by flow cytometry using CytExpert v2.4 software (Beckman Coulter):

Table 4 List of Errors Incurred by Using Flow-Count Fluorospheres

Factor	Actions required
Incorrect dispensing volume	With the reverse pipetting method, dispense beads at room temperature using the same calibrated pipet and tips used for the blood sample. Avoid air bubble formation. Wipe the pipet tip with paper starting from the base and avoid touching the tip hole.
Incorrect bead concentration	The bead bottle must be stored in an upright position tightly to avoid evaporation and beads leakage. Do not aliquot. Dispense beads after proper mixing and acquire immediately to avoid new sedimentation.
Incorrect storage condition	The aluminum foil must be removed entirely after the first opening. The opened bottle is stable for at least 30 days if properly stored. Be careful not to extend the time at room temperature longer than necessary. Avoid even accidental freezing of beads by placing the bottle away from the refrigerator rear wall, mainly if no internal ventilation is provided.

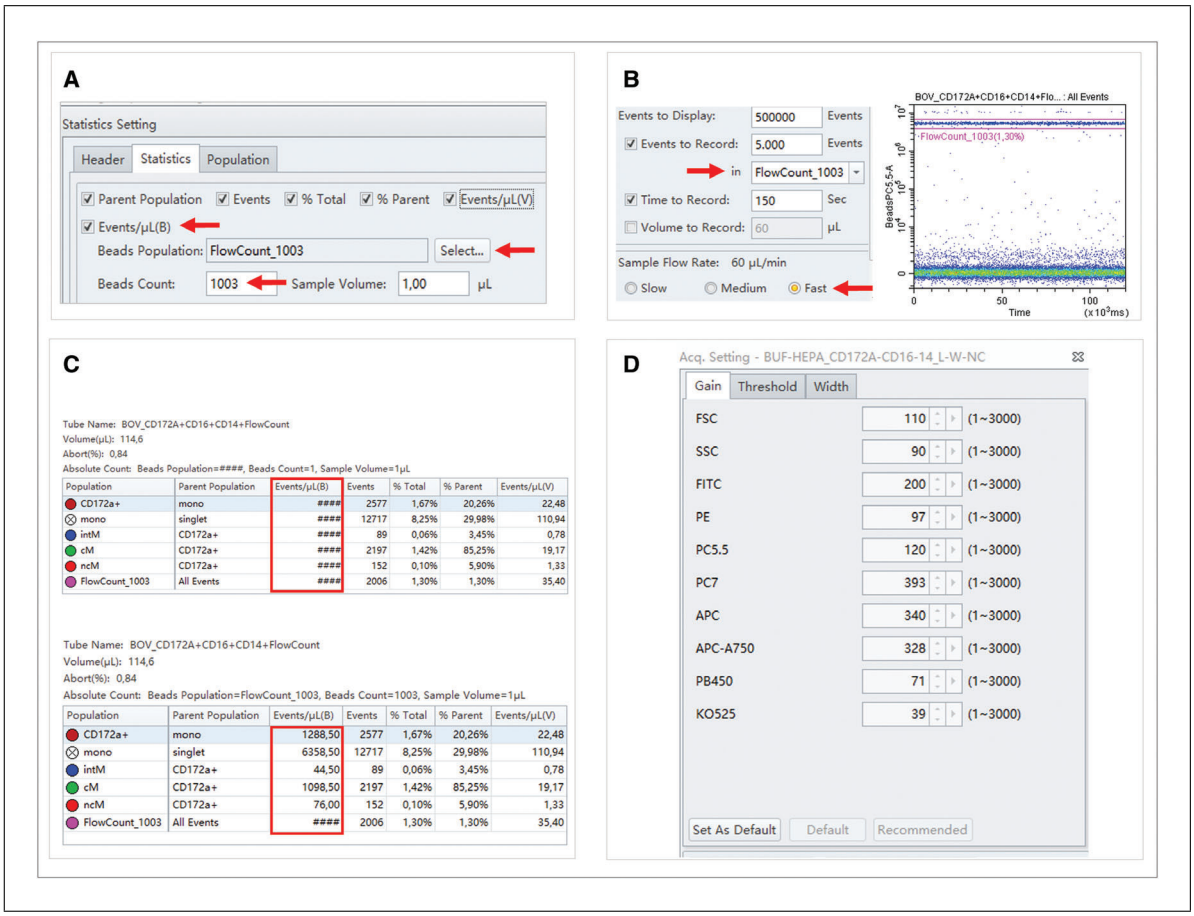

Figure 1 CytExpert v2.4 software settings (red arrows) for correct acquisition and automatic calculation (red boxes) of Flow-Count beads (**A-C**). Channel settings (**D**).

a. In the "Statistic settings" form: (a) enable "Events/µl(B)" counting type; (b) select the gated "Bead Population"; (c) insert the correct Flow-Count assayed concentration (from manufacturer's Assay sheet; Fig. 1A).
b. Set sample flow rate at 60 µl/min (Fast mode), and apply the stopping rule at least 3000 counting bead events in the gate Flow-Count on dot plot Time versus beads/All events (Fig. 1B).
c. Mix briefly the FC-tube and acquire it immediately.
d. Automatically, the acquisition software calculates the monocyte subset absolute counts (Fig. 1C). Channel settings are shown in Figure 1D. The adopted gating strategy is described in Figure 2.

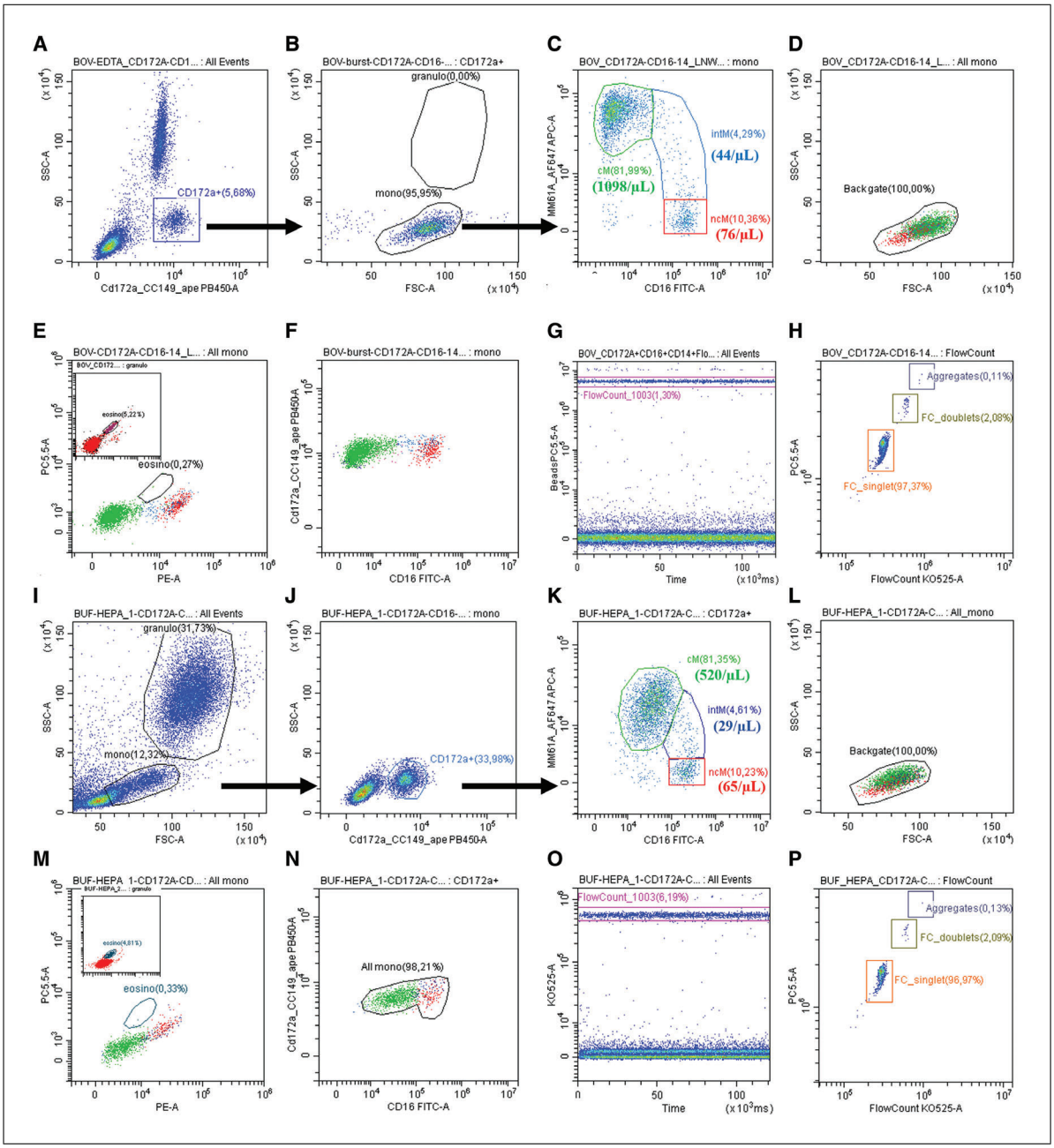

Figure 2 Gating strategy for bovine (**A-H**) monocyte subset absolute counts. (A) The dot plot CD172a versus SSC/All Events (= ungated) is used to identify all bovine monocytes as $CD172A^+$ cells. (B) The light scattering dot plot FSC versus SSC/$CD172a^+$ is used to draw the gate "mono" excluding damaged (left) or aggregate (right) monocytes. (C) The dot plot CD16 versus CD14/mono is used to identify and enumerate cM, intM, and ncM. The Boolean gate "All mono" ("cM or intM or ncM"; from dot plots C) is used for back gating shown in the dot plots D-F. (D) The back gate confirmed the lowest light scattering of ncM. (E) The back gate is used to exclude possible contamination of eosinophils with low SSC. (G) The gate "FlowCount," applied to the dot plots (H) is drawn (not shown) to include doublets and aggregates, allowing the percentage control of doublets and aggregates. (**I-P**) The same gating strategy was used for bubaline monocyte subset absolute counts. As shown in Figure 8, the CD14 and CD172a staining levels are rather heterogeneous in buffalo. Therefore, in samples with low levels, it can be useful to invert the first two steps of the previous gating strategy. (I) The dot plot FSC versus SSC/All events is used to draw the gate "mono," which applied to the CD172a versus SSC dot plot allows all monocytes to be identified as $CD172a^+$ (J) excluding all lymphocytes.

e. Wash sample line with filtered Milli-Q grade distilled water at least 15 s before acquiring a new specimen.

Each lot of Flow-Count fluorospheres has a specific concentration of fluorospheres; make sure you have entered the correct one. To prevent carry-over errors, acquire a filtered water sample between counting specimens and check the absence of events in the gates: "beads," "cM," "intM," "ncM". Alternatively, restart the new sample acquisition 15-20 s from the beginning.

ALTERNATE PROTOCOL

ABSOLUTE COUNT OF cM, intM, AND ncM FOR SINGLE LASER PLATFORM

Neither consensus recommendations nor quality programs for veterinary clinical FC analysis have been established. For that reason, an FC interest group was formed at the contact site of the Italian Society for Cytometric Cell Analysis (ISCCA) and the European Society for Clinical Cell Analysis (ESCCA). One of the several surveys and initiatives of this FC interest group establishes that single-laser instruments and indirect fluorescence procedures are still widely used in livestock and veterinary laboratories. The panel designed involves only core markers directly labeled and excitable using only a blue laser source. The careful compensation protocol, particularly CD16-FITC versus CD14-PE, is essential to ensure the accurate determination of the ncM subset ($CD14^{-/low}CD16^{++}$). In addition, we recommend performing the fluorescent minus one (FMO) control to position gates and quadrants correctly.

Additional Materials *(see also Basic Protocol)*

Multicolor mAb cocktail (Panel B): CD14-PE/CD16-FITC/CD172a-PE-Cy5 (see recipe, and Tables 1 and 2)

All the required steps and practical considerations are common to the Basic Protocol.

SUPPORT PROTOCOL 1

IN-HOUSE MONOCLONAL ANTIBODY LABELING USING A PACIFIC BLUE™ KIT

The flow cytometric applications in immune system studies are considerably lower in veterinary than in human medicine. The reasons can be attributed to the lower economic resources and, in particular, the limited number of conjugated mAbs. Recently, this problem has been partly overcome using commercial kits for covalent in-house mAbs conjugation, switching protocols from indirect fluorescence to direct fluorescence procedures, and facilitating the multicolor FC panel design.

Materials

Anti-bovine CD172a, clone DH59B: 1 mg/ml [Washington State University-Monoclonal Antibody Center (WSU-MAC), cat. no. BOV2049; Table 2]
APEX™ Antibody Labeling Kits–Pacific Blue™ (Thermo Fisher Scientific, cat. no. A10478)
VersaComp Antibody Capture Kit (Beckman Coulter, cat. no. B22804) or equivalent

Three single-channel pipets (1-10 µl; 10-100 µl; 100-1000 µl) with sterile disposable tips (preferably with filter)
Polypropylene microtubes (0.2, 0.5, 1.5, or 2.0 ml)
Polypropylene 75- × 12-mm flow cytometer tubes, preferably with cap (e.g., Sarstedt, cat. no. 55.526.006)
Appropriate tube racks
Timer
Vortex mix (optional)

1. Tap antibody labeling tip (Component B) on a hard surface.

   WSU-MAC antibodies are produced in ascitic fluid and clarified by filtration (0.2 µm). APEX™ kits use a solid-phase labeling technique that captures the mAb (only IgG) on the resin inside the APEX™ antibody labeling tip. Any contaminants are then eluted through the tip.

2. Remove both caps from the labeling tip, then place them into a clean microcentrifuge tube (1.5 ml).

3. Hydrate resin in the antibody labeling tip by pipetting 100 µl wash buffer (Component C, yellow cap). Use the elution syringe (Component H) to remove excess wash buffer.

   The hydrated resin bed volume is 10-15 µl; therefore, removing 85-90 µl of wash buffer is necessary. Apply the antibody labeling tip to the elution syringe and gently push the wash buffer through the tip into the 2.0-ml microcentrifuge tube (waste tube). Pay attention to the reconstitution of the resin bed. The easy formation of air bubbles could compromise the monoclonal labeling and the elution steps.

4. Apply 10 µl (10-100 µl pipet) of anti-CD172a to the top of the resin bed. Gently push antibody solution onto the resin using the elution syringe and discard eluting buffer into 2.0-ml waste tube.

   Place the dispensing tip on top of the resin; avoid touching it so as not to compromise the resin bed.

5. Add to reactive dye vial (Component A, blue cap): 2 µl DMSO (Component D, green cap) and 18 µl Labeling Buffer (Component E, white cap) to dissolve dye spotted at the vial bottom.

   Take care to expose the fluorochrome to light for the shortest time. Pipet up and down seven to ten times after adding DMSO and five to seven times after Labeling Buffer.

6. Apply 10 µl of the 20-µl (10-100 µl pipet) reactive dye to the top of the resin bed. Push solution onto the tip using the elution syringe and discard buffer eluted in the waste tube.

   Take care to expose the fluorochrome to light for the shortest time.

7. Incubate tip for 2 hr at room temperature in the dark.

8. Wash antibody labeling tip twice with 50 µl Wash Buffer (Component C, yellow cap), pushing through the tip using the elution syringe into the waste tube.

   Pay attention to exposing the fluorochrome to light for the shortest time.

9. Add 10 µl neutralization buffer (Component F, purple cap) to a 0.5-ml microcentrifuge tube.

10. Apply 40 µl elution buffer (Component G, red cap) to the top and center of the resin. Position antibody labeling tip on the 0.5-ml microcentrifuge tube. Elute using the syringe. Vortex briefly.

    Avoid photobleaching of the labeled antibody by protecting the tube with aluminum foil.

11. In a new 0.2-ml tube, add 6.5 µl DPBS and 1.5 µl labeled mAb. Pipet up and down two to three times and vortex briefly.

    Perform steps 11-14 to check the labeling reaction. To date, no manufacturer's protocols are available. We suggest using compensation beads as a simple and cost-effective protocol.

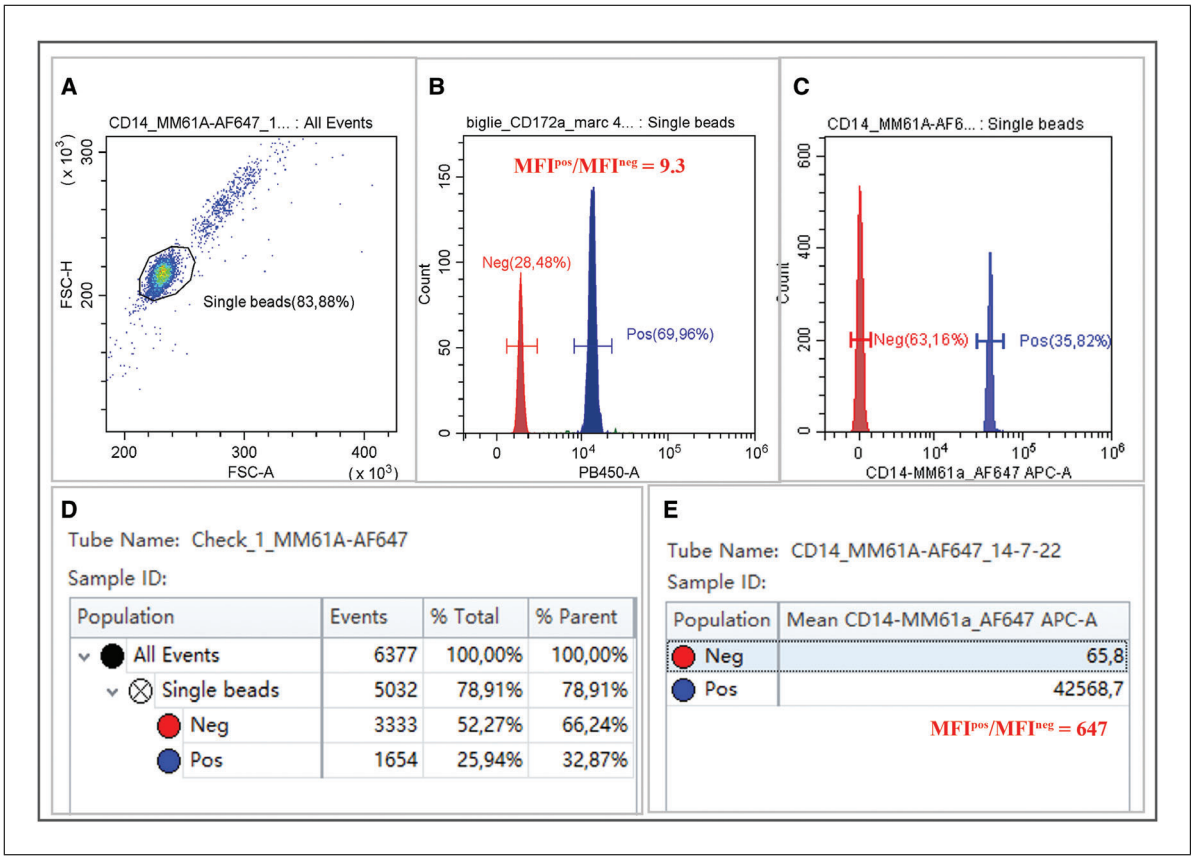

Figure 3 Conjugation efficiency test using VersaComp antibody capture beads. (**A**) Selection of single beads. (**B**) Example of APEX™ antibody labeling kits–Pacific Blue™ conjugation (see Support Protocol 1). (**C**) Example of Alexa Fluor® 647 antibody labeling kit™ conjugation (see Support Protocol 2). (**D**) Population hierarchy, and (**E**) mean fluorescence intensity (MFI) values of anti-CD14 AF647 conjugated.

12. Dispense ten drops of negative and positive VersaComp Antibody Capture beads in a 2-ml polypropylene microtubes. Vortex briefly.

    The bead manufacturer's protocol is to use one drop (~50 µl) for each type. This step saves ~40% of reagents, significantly reducing the test cost.

13. Mix 30 µl of each negative and positive bead sample in a flow cytometry tube with 8 µl from step 11. Vortex briefly and incubate at room temperature for 20 min in the dark.

14. Add 1 ml DPBS and centrifuge 300 × *g* for 6 min. Resuspend with 600 µl DPBS and acquire by flow cytometer using the channel settings shown in Figure 1D.

    Create a first gate around the single beads (Fig. 3A); then, using the respective gates, calculate the MFI^{pos}/MFI^{neg} ratio (Fig. 3B; where MFI is mean fluorescence intensity) to estimate the mAb conjugation efficiency. Usually, it ranges between 8 and 15 and the conjugation is stable for 4-6 months. Repeat the check monthly before use.

SUPPORT PROTOCOL 2 IN-HOUSE MONOCLONAL ANTIBODY LABELING USING AN ALEXA FLUOR® 647 KIT

Although anti-CD14-APC/AF647 are commercially available (Table 6), the authors consider in-house labeled clone MM61A still preferable (see Critical Parameters, Assay design section).

Additional Materials *(also see Support Protocol 1)*

Anti-bovine CD14, clone MM61A: 1 mg/ml (WSU-MAC, cat. no. BOV2109; Table 2)

Alexa Fluor® 647 Antibody Labeling Kit (Thermo Fisher Scientific, cat. no. A20186)
Milli-Q grade distilled water

Centrifuge with swing-out rotor for 5-ml tubes

1. Add 1 ml distilled water to the vial of sodium bicarbonate (Component B). Vortex or pipet up and down until fully dissolved.

   Complete bicarbonate dissolution takes a few minutes (10-20 min), by vortexing repeatedly. The solution can be stored at 2°C to 8°C for 1 month or frozen at −20°C for more extended storage. When using the frozen solution, ensure that it has reached room temperature and that there are no precipitates.

2. Add 9.1 µl $NaHCO_3$ to 91 µl anti-bovine CD14. Pipet up and down three to five times.

3. Transfer 100 µl of mAb-$NaHCO_3$ solution from the previous step to the vial of reactive dye. Pipet up and down seven to ten times to fully dissolve the dye.

   Avoid photobleaching of the labeled antibody by protecting the vial with aluminum foil.

   Put the purification resin flask to room temperature.

4. Incubate vial 1 hr at room temperature in the dark and mix gently every 10-15 min.

   Mix by small strokes with the finger at the bottom of the vial. Alternatively, pipet up and down five to seven times. Avoid vortexing. During this step, mix the purification resin flask manually by inverting several times and, ∼5 min before the end, prepare the spin column (next step).

5. Prepare spin column by removing the top cap, then the yellow cap; insert the second frit into the column and gently push it into contact with the one at the bottom of the column. Next cut off the square tab at the bottom of the column.

   Use the 10-100-µl pipet with a new tip to push the second frit.

6. Place spin column in a polypropylene 75- × 12-mm tube, add 1.0 ml resin, and allow it to settle by gravity.

   Allow the column buffer to drain by gravity. Initially, some pressure may be required to elute the first drops of buffer.

7. Add purification resin until the resin bed volume is ∼1.5 ml.

   Up until now, this level is not marked on the column, so consider 2 mm above the column widening. Alternatively, pipet a total of 1.55 ml of purification resin.

8. Centrifuge column at 1100 × *g* for 3 min using a swinging bucket rotor.

   A fixed angle rotor could be used so ensure the column is placed with the same orientation during the second centrifuge step.

9. Place spin column in a new 75- × 12-mm tube. Load the 100-µl reaction volume (from step 3) dropwise onto the center of the spin column. Allow solution to absorb into the resin bed.

   Expose the fluorochrome to light for the shortest time.

10. Centrifuge column at 1100 × *g* for 5 min.

11. Transfer purified labeled antibody eluted at the bottom of the tube (∼130 µl, mAb concentration: ∼0.5 mg/ml) to a 0.5-ml tube.

    Avoid photobleaching of the labeled antibody by protecting the tube with aluminum foil.

12. Check the labeling efficiency as shown in steps 11-14 of Support Protocol 1.

Usually, the MFI^{pos}/MFI^{neg} ratio ranges between 450 and 710 (Fig. 3C,E), and the directly conjugated mAb is stable for 7-10 months. Check the fluorochrome-mAb conjugation stability monthly or before use.

SUPPORT PROTOCOL 3

TITRATION OF FLUOROCHROME-CONJUGATED ANTIBODIES

Nonspecific antibody binding is crucial in FC applications, mainly using L/NW staining protocols. Although our L/W/NC protocol allows a reduction of nonspecific antibody binding, proper mAb titration remains a central point during the optimization protocol steps (Fig. 4). The mAbs titration has been extensively explained (Current Protocols article: Hulspas, 2010); here, we want to briefly report practical solutions to performing this procedure.

Materials

Fluorochrome-conjugated mAb (Table 2)
Bovine and water buffalo anticoagulated blood samples
Tris-buffered ammonium chloride lyse solution
DPBS without Ca^{2+} and Mg^{2+} plus 1% BSA (see recipe)

Polypropylene microtubes (0.2 ml)
Polypropylene 75- × 12-mm flow cytometer tubes
Two single-channel calibrated pipets (2-20 µl; 100-1000 µl) with sterile disposable tips (preferably with filter)
Vortex
Timer

1. Prepare eight microtubes (C1-C8) by adding DPBS, as shown in Figure 5A.

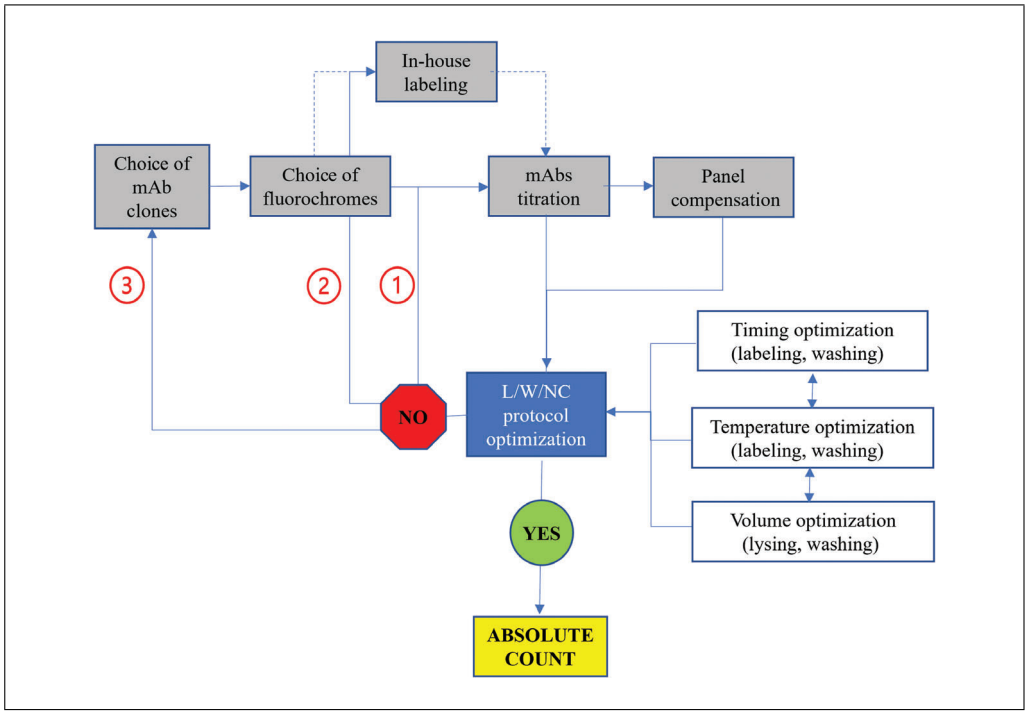

Figure 4 Flowchart for design (gray boxes) and optimizing (white boxes) of a multicolor flow cytometry (FC) protocol using a lyse/wash/no-centrifugation procedure. Should the optimization of labeling, wash, and lyse steps be insufficient to achieve the correct labeling of monocyte subsets, proceed to modify the design steps in the order shown by the red numbered circles.

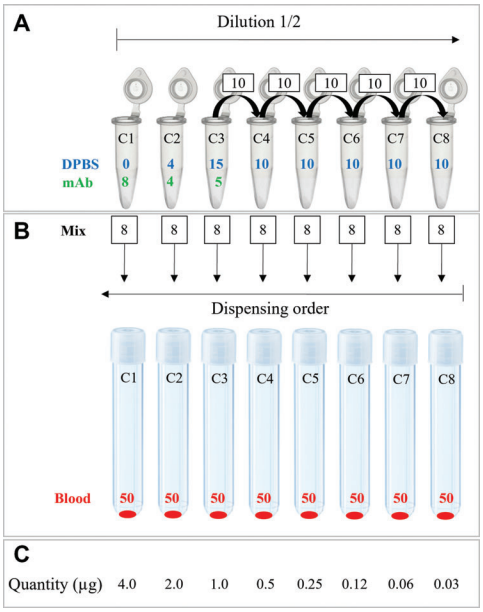

Figure 5 Schematic representation of the titration procedure (see Support Protocol 3). (**A**) Preparation of C1-C8 serial dilutions and (**B**) blood staining. (**C**) Purely by way of example, only quantities of anti-CD14 AF647 are conjugated. All DBPS, mAb, mix, and blood volumes are expressed in microliters.

The C1 microtube can be omitted and the volume of mAb can be added directly into the 75- × 12-mm tube (step 7). Close the tube immediately after adding the DPBS to avoid losses due to evaporation. We recommend using eight concentrations, diluting 1/2 (1 part of 2 parts total).

2. Add the volume of mAb to microtubes C1-C3 (Fig. 5A); vortex briefly.

   Generally, 8 µl of mAbs shown in Table 2 is the correct amount to prepare the start serial dilution C1.

3. Add 10 µl C3 mix to C4 tube (with 10 µl DPBS), cap, and vortex briefly.

4. Using the same tips, pipet up and down two to three times and repeat step 3 to prepare dilution C5.

5. Repeat the previous step to prepare dilutions C6-C8.

6. Pipet 50 µl anticoagulated blood at the bottom of the flow cytometer tubes (C1-C8, Fig. 5B).

7. Add 8 µl of DPBD plus mAb mix, starting from C8, to the corresponding dispensed blood sample (Fig. 5B); vortex briefly.

   Add the mAb plus DPBS mixture to the blood in the order C8 → C1. This method reduces nonspecific binding due to highly concentrated mAb (C1-C3).

8. Follow steps 3-5 of Basic Protocol.

9. Acquire data by flow cytometer using the same acquisition protocol used for counting (see Basic Protocol and Fig. 1).

10. Choose the saturating concentration with the highest signal-to-noise ratio (S/N).

    For more details, refer to Current Protocols article: Hulspas, 2010.

REAGENTS AND SOLUTIONS

Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} and Mg^{2+} plus 1% BSA

- Dulbecco's Phosphate Buffered Saline (DPBS), without Ca^{2+} and Mg^{2+}, liquid, sterile-filtered (MilliporeSigma, cat. no. D8537)
- BSA solution, 10% in DPBS, sterile-filtered (MilliporeSigma, cat. no. A1595)

Use a laminar flow cabinet to add, in a sterile 50-ml polypropylene tube, 45 ml of DPBS and 5 ml of 10% BSA. Withdraw the required volume maintaining the solution sterility.

Store at +4°C for up to 3 months.

Tris-buffered ammonium chloride lyse solution

- 0.88 % (w/v) NH_4Cl
- 7.93 mM Tris base (CAS number: 1185-53-1)
- Milli-Q grade distilled water
- 1 M HCl

Dissolve 8.75 g NH_4Cl and 1.25 g Tris base in ~950 ml Milli-Q grade distilled water in a volumetric flask. Adjust pH to 7.2-7.4 with 1 M HCl then adjust the final volume to 1 L. Aliquot into bottles and sterilize by autoclaving on the liquid cycle (121°C, 20 min). Aliquot the cooled sterilized solution into 50-ml Falcon-type tubes and store at −20°C for up to 12 months. Use the lysis solution at room temperature.

Store remaining solution at +4°C for ~3 weeks and keep tightly closed.

CAUTION: Hydrochloric acid is highly corrosive; handle it carefully while complying with local safety regulations.

Multicolor mAb cocktail (Panel A)

- Titrated anti-bovine CD14, AF647 in-house labeled, clone MM61A (see Support Protocols 2 and 3)
- Titrated anti-bovine CD16, FITC, clone KD1 (Bio-Rad, cat. no. MCA5665F; see Support Protocol 3)
- Titrated anti-bovine CD172a, Pacific Blue in-house labeled, clone DH59B (see Support Protocols 1 and 3)
- DBPS without Ca^{2+} and Mg^{2+}

Prepare the mAb cocktail daily. To the 0.2-ml tube, add DPBS to a final volume of 8 µl/sample. Add mAb quantities detected by the titration tests. Vortex briefly and gently. The remaining can be stored at +4°C for a few days. Protect from light and check the in-house labeling stability before reuse.

Purely by way of example, data shown in Figure 2 are obtained using this mAb cocktail (for five samples): CD14 = 1.5 µl; CD16 = 7.5 µl; CD172a = 4.0 µl; DPBS = 27.0 µl.

Multicolor mAb cocktail (Panel B)

- Titrated anti-bovine CD14, PE, clone CC-G33 (Bio-Rad, cat. no. MCA2678PE)
- Titrated anti-bovine CD16, FITC, clone KD1 (Bio-Rad, cat. no. MCA5665F)
- Titrated anti-bovine CD172a, PE-Cy5, clone CC149 (Bio-Rad, cat. no. MCA2041C)
- DBPS without Ca^{2+} and Mg^{2+}

Prepare the mAb cocktail daily. To the 0.2-ml tube, add DPBS to a final volume of 12 µl/sample, then the mAb quantities detected by the titration tests. Use the same precautions as described in the Multicolor mAb cocktail (Panel A) recipe.

Purely by way of example, data shown in Figure 2 are obtained using this mAb cocktail (for five samples): CD14 = 40.0 µl; CD16 = 7.5 µl; CD172a = 10.0 µl; DPBS = 2.5 µl.

COMMENTARY

Background Information

The existence of several subsets within the circulating monocyte population was first reported by Passlick and co-workers (Passlick, Flieger, & Ziegler-Heitbrock, 1989). Human blood monocytes are currently classified into $CD14^{++}CD16^{-}$ classical monocytes (cM), $CD14^{++}CD16^{+}$ intermediate monocytes (intM), and $CD14^{+}CD16^{++}$ non-classical monocytes (ncM; Ziegler-Heitbrock et al., 2010). Monocyte heterogeneity has also been reported in several veterinary species such as cattle (Hussen et al., 2013), sheep (Elnaggar et al., 2016; Pridans et al., 2016), pigs (Fairbairn et al., 2013; Moreno et al., 2010), camels (Hussen et al., 2020), and dogs (Gibbons et al., 2017). In 2013, Hussen and co-workers reported the existence of three different monocyte subsets in bovine blood (Hussen et al., 2013). Similar to humans, bovine cM are $CD14^{++}CD16^{-}$, and bovine intM are $CD14^{++}CD16^{+}$. Bovine ncM, instead, showed high CD16 with absence or very low CD14 expression ($CD14^{-/low}CD16^{++}$; Hussen et al., 2013). Further studies characterized the subset-specific immunophenotypic and functional properties (Corripio-Miyar et al., 2015; Grandoni et al., 2021; Hussen & Schuberth, 2017; Hussen et al., 2013; Talker et al., 2022; Talker et al., 2018). Like their composition in humans, bovine monocytes consist of a major fraction (~90%) of cM and two minor populations of intM and ncM (5% to 10% for each subset; Hussen et al., 2013). In contrast, insights on buffalo monocyte subsets are limited to the co-expression of core markers and CD163 on leukocytes (Elnaggar et al., 2019). This is due to the lack of buffalo species-specific mAbs; this renders necessary that the cross-reactivity of mAbs from other animal species, including humans, is exploited. Although some gaps remain, such as the lack of anti-CD45 and the need to use CD18 as a pan-leukocyte marker, most anti-bovine mAbs cross-react with buffalo orthologous molecules (Grandoni et al., 2017). So we consider it mandatory to use cattle as a control during the setup of buffalo protocols. Considering the immunophenotype of the three subsets in cattle and buffalo species, CD14 does not allow the detection of all monocytes. Therefore, the best choice is using CD172a, a member of the signal regulatory protein (SIRP) family, which is a marker for all myeloid cells.

The absolute quantification of lymphocyte subsets has always been crucial in several clinical situations in humans. The first major challenge was, in the early 1980s, the $CD4^{+}$ T lymphocyte count in patients with HIV. Mandy and Brando (2001; Current Protocols article) have well illustrated the absolute count problems in humans, highlighting critical issues and proposing practical strategies to overcome them. Absolute counts of cell subsets can be performed directly (single platform) or indirectly (dual platform). The first method counts subpopulations (e.g., cM, intM, ncM) using only a flow cytometer. In the second method, their absolute counts are obtained using number/µl of total monocytes, using a hematology analyzer, multiplied by the flow cytometer's subset percentages. However, the single-platform method should be considered the gold standard for leukocyte subpopulation absolute counts, as shown by the European Working Group on Clinical Cell Analysis (EWGCCA; Gratama et al., 1998). Therefore, the use of L/NW staining protocols is highly recommended accordingly (Brando et al., 2000; Szaloki & Czeti, 2021).

Critical Parameters

Assay design

Figure 4 shows the flowchart for design (gray boxes) and optimizing (white boxes) of the proposed L/W/NC procedures. The availability of anti-CD16 is restricted to only one clone (KD1) used to identify the human orthologue. Both anti-CD172a mAbs work well, but only one labeling is available. Instead, several solutions are possible for the choice of anti-CD14. All mAbs work similarly; however, we preferred two anti-bovine clones (CC-G33 and MM61A) because their reduced nonspecific binding allowed faster optimization of the labeling protocol. For the complete list of available mAbs, see Table 6. It is important to highlight that the proposed protocols are specific to the buffalo river type. Eventual problems with samples of buffalo swamp type cannot be excluded, as evidenced by the lack of anti-CD4 clone CC8 cross-reactivity (Grandoni et al., 2020).

L/NW versus L/W/NC procedure

When performing a single-platform absolute count protocol, it is essential to minimize cell loss caused particularly by lysis and

sample washing steps. Usually, the washing steps (needed to remove or minimize the nonspecific binding of mAbs) involve the addition of an isotonic solution (e.g., PBS, sheet fluid), centrifugation, and resuspension of the labeled cell pellet. Among these, centrifugation represents the crucial step. In the proposed protocols, by adding and incubating cold DPBS plus 1% BSA to the lysed sample, we obtain simultaneously:

- reduced leukocyte damage due to lysis solution;
- a significant reduction of mAb nonspecific bindings (see Fig. 6 and Table 5);
- avoid cell loss by centrifugation;
- minimize doublets and multiple aggregate formations.

Therefore, because the centrifugation step is omitted, we call this procedure lyse/wash/no-centrifugation (L/W/NC) instead of L/NW.

Optimizing L/W/NC procedure

The steps that need to be modified are represented in Figure 4 (white boxes). Incubation times and temperatures of labeling, lysis, and washing steps are factors closely related. In the optimization phase, we evaluated the combinations of:

- four incubation times (10, 15, 20, 30 min) and two temperatures (+4°C, room temperature) for the labeling step;
- two incubation times (5, 10 min) and two temperatures (+4°C, room temperature) for the washing step;
- two different volumes (0.5, 1.0 ml) for erythrocyte lysis and leukocyte washing.

Our criterion for evaluating the proper optimization of the L/W/NC procedure was choosing the combination of factors with the highest signal-to-noise ratio (S/N).

If adequate optimization cannot be achieved, it will be necessary to redesign the assay by acting on the general steps (gray boxes) in the priority order proposed.

Proper titration of mAbs is the first element to act on to minimize nonspecific binding.

Accurate and reproducible pipetting

In a single-platform absolute counting protocol, the proper dispensing of blood and fluorescent counting beads is essential to obtain accurate and reproducible results. Therefore, applying the reverse pipetting technique, described by Brando et al. (2000), is essential. Here, we want to add additional pointers that can help increase this step's performance. Usually, air-displacement pipets are used in flow cytometry laboratories; refer to the instructions provided by the pipet manufacturer.

Briefly, for the correct execution of reverse pipetting:

(1) Holding the pipet vertically, press the plunger down to the second stop. Wait 1 to 2 s;

(2) Immerse the pipet tip in the blood sample. Allow the plunger to move up to the rest position;

(3) Tilting the pipet (30 to 40°) and dispensing the sample volume at the bottom of the labeling tube, push the plunger to the first stop position. Wait ∼1 to 2 s.

Use the same calibrated pipet and only low-retention original or recommended tips. Employ commercial ones only if previously tested with the gravimetric analysis (considering blood density is 1.08 g/ml). In addition, the type of pipet tip should be selected so that the air cushion between the pipet piston and the liquid surface is as small as possible. With the reverse pipetting, the volume aspirated will be greater than the volume dispensed, and the excess will be discarded. Consider a dead volume of counting beads about 14 µl, 30 µl, and 75 µl by using a 5- to 50-µl, 10- to 100-µl or 20- to 200-µl pipet, respectively. The purchase of a fixed-volume pipet (50 µl) may be worthwhile considering the reduced purchase and calibration price.

The volume of mAb cocktail, lysis solution, and PBS does not affect the absolute count; therefore, the forward pipetting method can be used. Table 3 lists the factors affecting pipetting accuracy and reproducibility (precision). It is important to remember that although the effect of a single factor could be small, the error may be significant when several factors are added together.

Correct use of fluorescent counting microbeads

Fluorescent counting microbeads are needed to perform absolute counts using instruments, such as CytoFlex, that employ flow rate-based cytometry. However, their incorrect use significantly affects the accuracy and reproducibility of results. The main errors found using suspended count beads are (1) the resuspension step, (2) volume dispensing, (3) the gating strategy used to identify them, and (4) carry-over from the previous sample.

Suspended particles tend to aggregate; unfortunately, counting beads and cells are no exception, forming doublets and multiple aggregates between beads, cells, and cell-bead. The result will be an underestimation of the

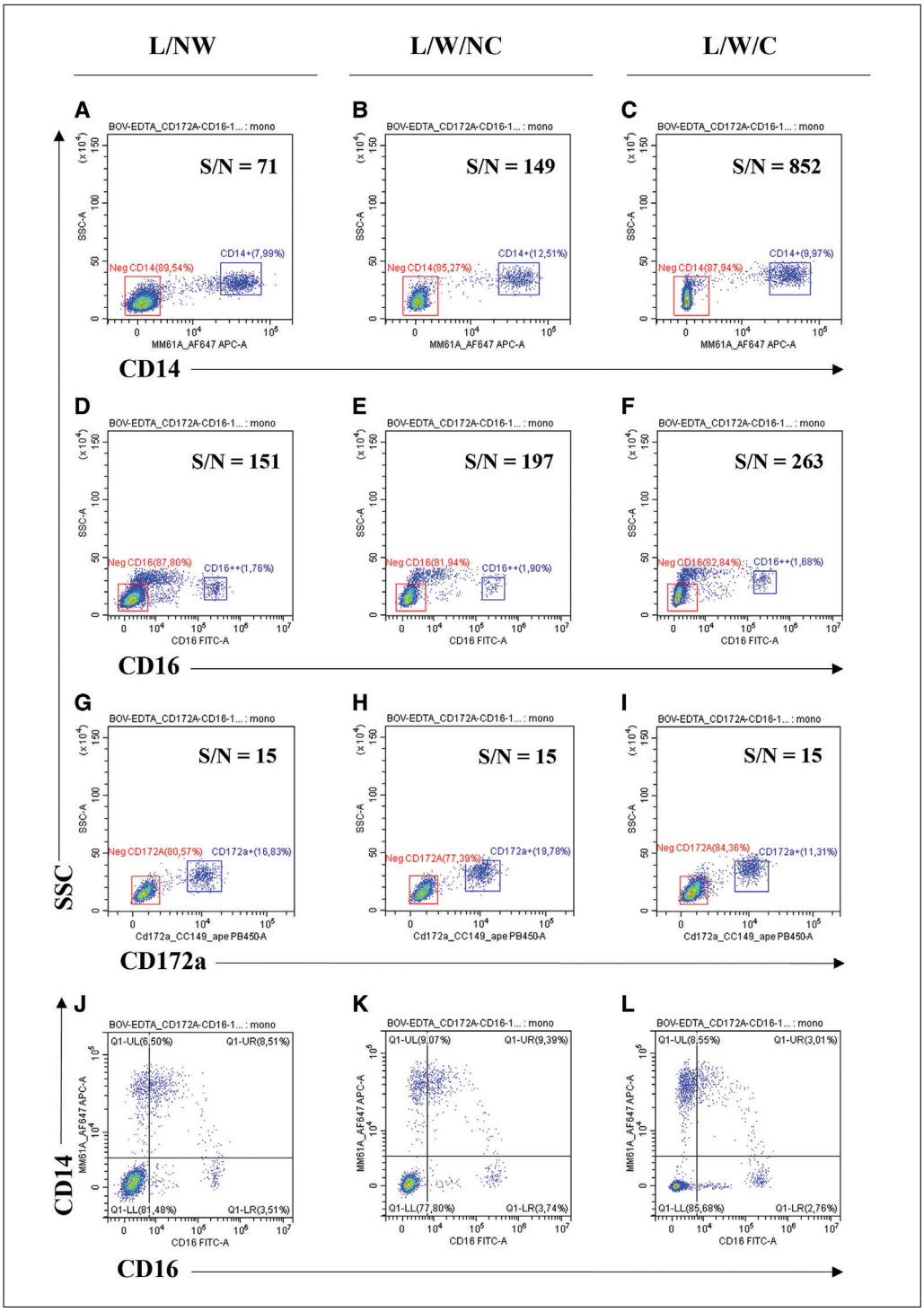

Figure 6 Effects of the three procedures on bovine blood labeling using anti-CD14 (**A-C**), anti-CD16 (**D-F**), anti-CD172a (**G-I**), and anti-CD16 vs anti-CD14 (**J-L**). The procedures of lyse/no-wash (L/NW), lyse/wash/no-centrifugation (L/W/NC), lyse/wash/centrifugation (L/W/C) are shown in the left, center, and right dot plots, respectively. The blue border gate was used for the positive population, the red border gate for the negative population. Samples obtained by the L/NW procedure were used to place gates and the quadrant. Abbreviation: S/N, signal-to-noise ratio.

subset counts. Bead-bead aggregation occurs, in particular, during storage of reagent, while bead-cell doublet formation occurs after bead addition to labeled cell samples. To avoid it, refer to the used product package inserts for specific directions on proper bead storage, resuspension, and specimen addition. See Table 4 for a list of main errors incurred using Flow-Count fluorospheres. When excited by a blue laser (488 nm), these counting

Table 5 Troubleshooting Guide

Problem	Possible cause	Solution
Appearance of two or more fluorescent bead populations	Incorrect mix of the bead bottle	Check in the dot plot time versus beads the flow stability and/or a possible bead aggregation during acquisition
	Sample carry-over	Acquire filtered distilled water to exclude sample carry-over
	Fluidic instability	Acquire a bead control in PBS to exclude bead deterioration
	Bead deterioration	Prepare sample again (starting with unlabeled blood); take care to mix the bead bottle and the sample correctly before dispensing and acquisition, respectively
Formation of cell doublets and multiple aggregates	Incorrect treatment of the sample	Increase the time and force of agitation of sample and beads
		Add EDTA to the wash buffer
	Intrinsic characteristic of the sample	Use new beads
Low S/N ratio	Low efficiency, in-house mAb labeling	Extend the mAb labeling time provided by in-house protocol to overnight incubations at 4°C after an initial incubation of 1 hr at room temperature
	Loss of mAb brightness	
	Incorrect titration of mAb	Increase amount of dye in the reaction by using the contents of two vials of reactive dye
		Check labeling stability of mAb using compensation beads (steps 11 or 12 of in-house labeling Support Protocols 1 and 2, respectively)
		Repeat the mAb in-house procedure; pay attention to preparing the antibody labeling tip or spin column of the in-house labeling Support Protocols 1 and 2, respectively
		Repeat titration procedure
Poor cell clusters separation	Inappropriate sample storage condition	Check that the sample storage time and temperature are respected
	Incorrect labeling conditions	Avoid using samples >4 hr after collection
		Do not use samples with an incorrect blood/anticoagulant ratio (e.g., tubes not totally filled)
	Insufficient washing of the sample	Check observance of times, temperatures, and volumes of labeling protocol
	Incorrect titration of mAb	Reduce time and strength of mixing
		Repeat titration procedure

Table 6 List of Monoclonal Antibodies Available for the Identification and Enumeration of Monocyte Subsets

Specificity	Clone	Labeling available	Mouse isotype	Target species	Reference to buffalo cross-reactivity[a]
CD14	CAM36A	Not available	IgG1	Bovine	Grandoni et al., 2017
	CAM66A	Not available	IgM	Caprine	Grandoni et al., 2017
	CC-G33	FITC, PE, AF647	IgG1	Bovine	This article
	M5E2	Complete	IgG2a	Human	Grandoni et al., 2022
	MM61A	Not available	IgG1	Bovine	Grandoni et al., 2017
	TÜK4	Complete	IgG2a	Human	De Matteis et al., 2016
CD16	KD1	FITC, PE, AF647	IgG2a	Human	Elnaggar et al., 2019
CD172a	CC149	PE-Cy5	IgG2b	Bovine	This article
	DH59B	Not available	IgG1	Bovine	Grandoni et al., 2017

[a] References to the FC applications. For references to bovine cross-reactivity, refer to Grandoni et al., 2021.

microbeads have a fluorescence emission range of 525 nm to 700 nm. They are performed for Cytomics FC 500 and Navios flow cytometry systems (Beckman Coulter); on CytoFlex, they are positioned at the fluorescence scale bright end. Then reduce the gain of the free channel used for the time versus beads dot plot to a helpful value for visualizing single and bead aggregates. Use a specific gate to calculate the aggregate percentage and evaluate the correct use of beads (see the Troubleshooting section). Finally, at least 2000 to 3000 Flow-Count single events (never less than 1000) must be acquired. Ensure fluidic stability using the instrument perfectly clean with the sheath fluid tank >50% full, avoiding air bubble formation. When ready for the acquisition, add counting beads to sample, then mix gently by vortex or with finger strokes.

Troubleshooting

Controlling the critical factors described above allows the two proposed counting protocols to be carried out quickly and easily. The factors that may represent meaningful problems are described in Tables 3, 4, and 5.

Understanding Results

Strategies for controlling the variables discussed in the previous section, gating strategies for identifying the three monocyte subsets, and interpreting the results are described here.

Optimizing of 3-color/zero compensation panel

Figure 6 compares the results obtained with the three different sample treatment procedures:

- Lyse/no-wash (L/NW);
- Lyse/wash/no-centrifugation (L/W/NC);
- Lyse/wash/centrifugation (L/W/C).

These procedures can be performed on the same sample for easier and faster optimization. For the L/NW procedure, acquire an aliquot (50 µl) after step 4 of the Basic Protocol. For the L/W/C procedure, centrifuge ($300 \times g$ for 5 min) the remaining sample after the L/W/NC acquisition, then acquire the resuspended cell pellet with 1 ml of DPBS plus 1% BSA. The highest S/N was used to choose the best conditions (mAb titrations, time and temperature labeling, lysis and washing volumes). As already demonstrated for immunophenotyping (Grandoni et al., 2021), K_3EDTA is also the preferred anticoagulant for absolute counts of bovine monocyte subsets (Fig. 7A-F). In fact, in addition to showing a significant reduction in CD163 labeling (Grandoni et al., 2021), the use of Li-heparin causes a worsening of monocyte light scattering parameters (Fig. 7D-E), hindering the differentiation from progenitor motor neurons (PMNs), which in some cases may show expression of CD14 (Sohn et al., 2007). In buffalo, on the other hand, K_3EDTA causes a decrease of CD172a (Fig. 7H versus 7K) and especially CD14 labeling (Fig. 7I versus 7L). Although Li-heparin may cause the same effect regarding the separation from granulocytes (Fig. 7J-K), to study total and subset monocytes, to date, it is the anticoagulant that has been more helpful.

Create gating regions and acquire data

In Figure 2 are shown the gating strategies used for identifying and counting bovine (Figure 2A-H) and water buffalo (Fig. 2I-P) monocyte subsets. The gating strategy used for cM, intM, and ncM identification alone has been described in our previous work (Grandoni et al., 2021). For absolute counting, however, it is necessary to consider that the major pitfalls are: (1) the inclusion of all events of interest and (2) the exclusion of all undesired events. This is easy to write, less so to achieve. As extensively explained, in the single-platform absolute count is needed to use counting beads. It is, therefore, mandatory to verify that only single bead events are included in the Flow-Count_1003 counting gate (Fig. 2G and 2O) as required by the manufacturer.

The dot plot Time versus Bead-PC5.5 allows control of fluidic stability and doublet bead exclusion at the same time. It is still necessary to check the percentage of double and aggregate bead events (Fig. 2H and 2P) to ensure the correct bead resuspension and treatment of the sample (Tables 4 and 5).

Likewise, it is crucial to include all monocytes and exclude other leucocytes (Fig. 2A-B and 2I-J). As shown in our previous works, bovine (Grandoni et al., 2021; Hussen et al., 2013) and bubaline monocytes are identified as $CD172a^+$ (Fig. 2A and 2J) cells, respectively. Instead, CD16 and CD14 expression patterns allow identification of cM, intM, and ncM. However, gate positioning is difficult when cell clusters are not well defined. In particular, intM (Fig. 2C and 2K) appears as a continuous subset between cM ($CD14^+/CD16^{-/low}$) and ncM ($CD14^{-/low}/CD16^+$). Furthermore, despite optimizing the L/W/NC procedure, non-specific labeling cannot be totally eliminated. However, gate drawing is more feasible, due to the

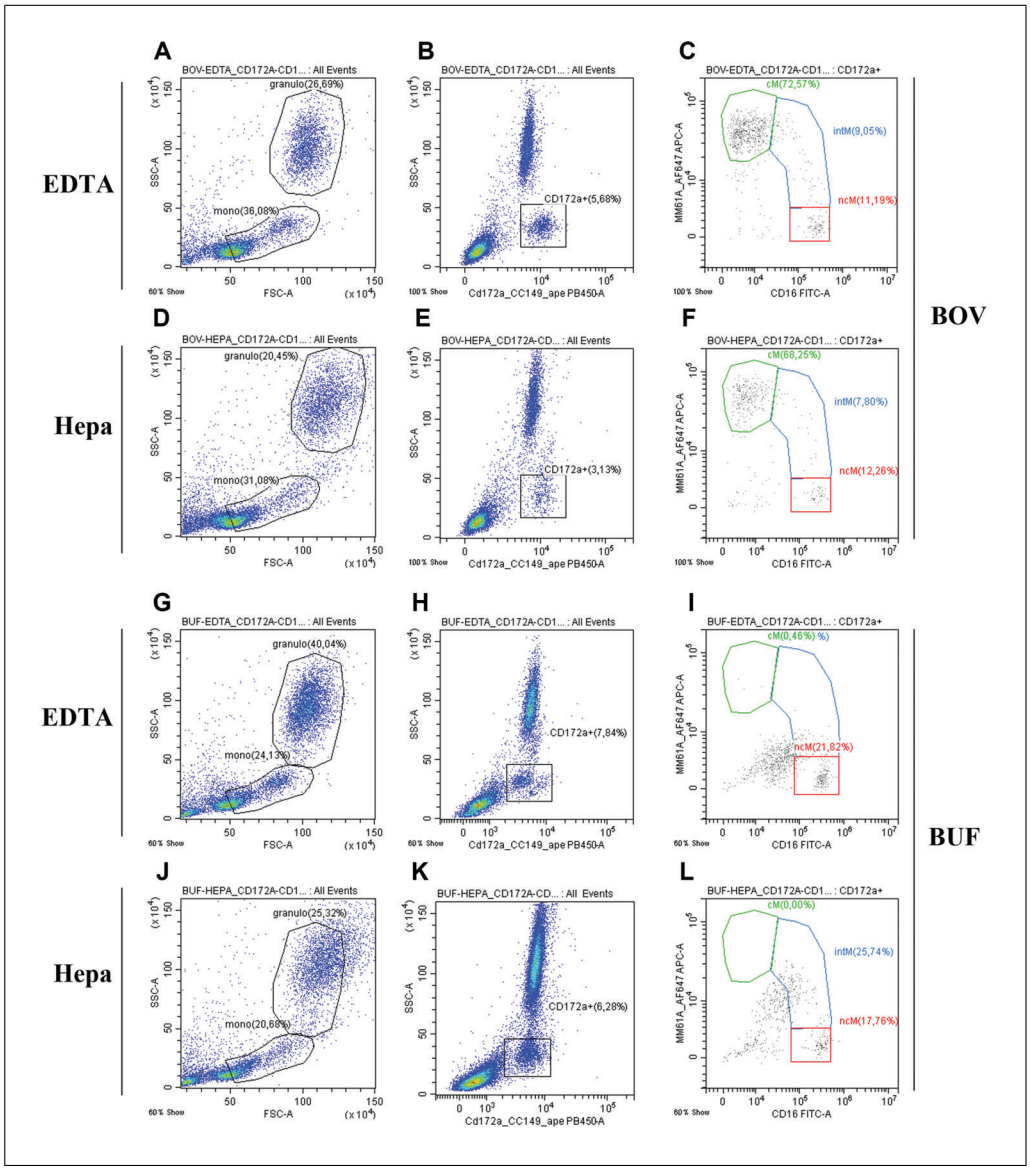

Figure 7 Effect of anticoagulants on light scatter parameters (left dot plots), CD172a staining (center dot plots), and monocyte subset identification (right dot plots) from bovine (**A-F**) and buffalo (**G-L**) blood samples. Abbreviations: BOV, bovine; BUF, buffalo; Hepa, heparin.

absence of spillover between fluorescent channels, by means of a zero compensation panel. In addition, for the correct ncM gate drawing ($CD14^{-/low}$), we recommend the CD14 FMO plus isoclonic control (Fig. 8A and 8B). Finally, after applying all appropriate controls, fixed gates should not be moved during the analysis of different samples.

In buffalo, due to different staining intensities of anti-CD14 and anti-CD172a mAbs, we suggest a different gating strategy. On the light scattering dot plot (FSC versus SSC; Fig. 2I), we draw a polygonal "mono" gate around a large region, including all monocytes. This gate was applied on CD172a versus SSC dot plot (Fig. 2J) to identify only monocytes as $CD172a^{+}$ cells, excluding all lymphocytes. For the first time, and as in other mammalian species, including humans, at least three monocyte subsets are also detected in water buffalo. Our three-laser/zero compensation panel allows the characterization of a bubaline cM, intM, and ncM monocyte subset as $CD14^{+/++}/CD16^{-/+}$, $CD14^{+/low}/CD16^{+/++}$, and $CD14^{-/low}/CD16^{++}$, respectively (Fig. 2K). Interestingly, cM showed a significantly different expression level of CD14 and CD16 antigens between cattle (Figs. 2C and 7C) and buffalo (Figs. 2K and 8D-G). Bovine cMs are mostly $CD16^{-}$ while bubaline cMs are mostly $CD16^{+}$. On the other hand, buffalo CD14 showed a heterogenic expression, ranging from dim to medium, compared to bovine levels. Although a lower affinity of

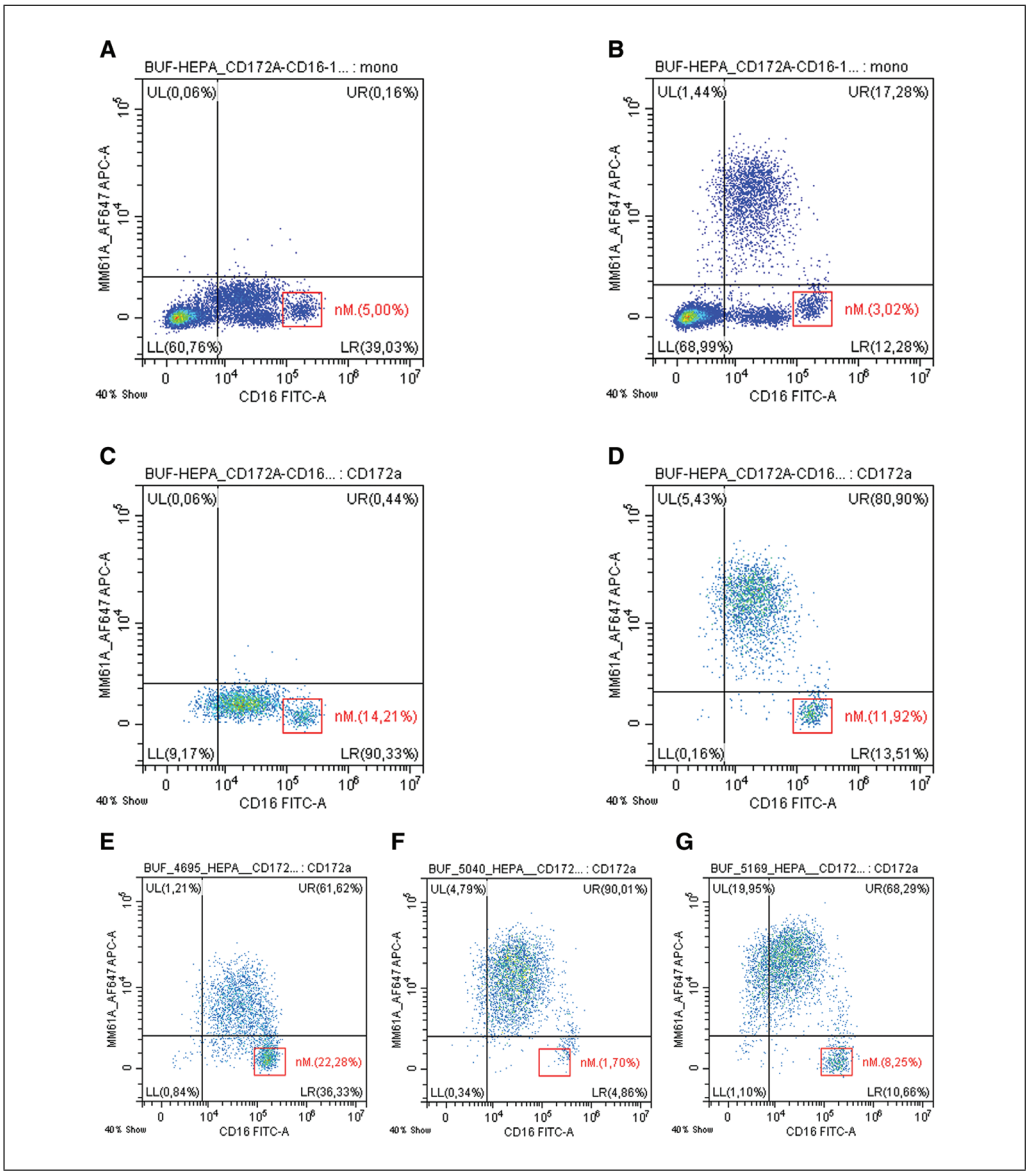

Figure 8 The fluorescent minus one (FMO) + isoclonic CD14 control (**A and C**) was obtained by labeling (10 min, room temperature) with 10× the saturating concentration of purified anti-CD14 (MM61A), followed by labeling with panel A (including MM61A-AF647). Dot plots (**B and D**) show the same specimen labeled by only panel A. Gates "mono" (including large lymphocytes, A-B) and "CD172A^{+}" (including only monocytes, C-G) were applied to the CD16 versus CD14 dot plots. Dot plots (**E-G**) were obtained from three other buffaloes.

cross-reactive anti-CD14 mAb and/or an effect by anticoagulant cannot be excluded, the differences in the results from presented buffaloes are undoubtedly evident.

The first proposed gating strategy can be used to analyze panel B, both bovine and buffalo samples. However, pay attention to the accurate protocol compensation and execution of FMO controls for gates and quadrant positions.

Time Considerations

The Basic Protocol and Alternate Protocol need ~40 min to complete labeling, lysis, and washing steps of four to six samples, including handling operations. The acquisition of 5000 Flow-Count events takes ~2 min. The results of the count are simultaneous with the sample acquisition.

The in-house mAb labeling described in Support Protocols 1 and 2 need ~3 and 1.5 hr, including handling and mAb labeling of the efficiency control, respectively.

The titration procedure needs ~1 hr to complete all steps (handling of eight dilution points, L/W/NC labeling protocol, sample acquisition, and identification of saturating concentration).

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Author Contributions

Francesco Grandoni: Conceptualization, data curation, formal analysis, writing original draft, writing review and editing; **Daniela Fraboni:** Supervision; **Barbara Canonico:** Supervision, writing review and editing; **Stefano Papa:** Supervision, writing review and editing; **Francesco Buccisano:** Supervision, writing review and editing; **Hans-Joachim Schuberth:** Supervision, writing review and editing; **Jamal Hussen:** Conceptualization, writing original draft, writing review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

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