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LETTER TO THE EDITOR-CASE REPORT

CLINICAL CYTOMETRY WILEY

Steric hindrance: A practical (and frequently forgotten) problem in flow cytometry

Steric hindrance (SH) is a long-term known, albeit often forgotten, phenomenon in which the reduction, or less frequently the absence, of detectable fluorescence signals occurs because one monoclonal antibody hinders the binding of another one to its respective target antigen, when the reagents are used conjointly. The mechanism underlying SH is at least partially related to the presence of multiple monoclonal antibodies targeting the same macromolecular complex. Therefore, with the aim to avoid SH, each monoclonal antibody combination to be implemented in flow cytometry's routine should be beforehand tested against experiments of single-color staining. This procedure is part of the validation's studies of antibody and fluorochrome optimization recommended by the International Council for Standardization of Haematology (ICSH) and the International Clinical Cytometry Society (ICCS).

Notwithstanding the checks for SH, some unexpected behaviors of monoclonal antibodies can be seen in ordinary samples, even when the procedure of staining is part of an already validated protocol used on daily bases in a flow cytometry laboratory.

In our own experience, during a period of 9 years (2009–2017) performing systematic flow cytometry assays, we found only one patient with the a diagnosis of chronic lymphocytic leukemia (CLL), in which the peripheral blood sample showed the presence of the SH phenomenon with the variable combination of the monoclonal antibodies anti-kappa (FITC), anti-lambda (PE), anti-CD19 (PE-Cy5) and anti-CD20 (PE-Cy5).

The patient was a 53-year-old man who, in 2014, presented with a white blood cell count of 76.5×10^9/L and a lymphocytes count of 52.8×10^9/L. The review of peripheral blood smear slide showed the presence of small to medium-sized mature-looking lymphoid cells.

For flow cytometry procedure, the lyse-wash technique was employed in a sample of peripheral blood collected in EDTA. Briefly, 1.0×10^9 leucocytes/L were incubated with 5 μl of each monoclonal antibody. The following immunophenotypic panel, that has been employed in our facility for the analysis of all suspected cases of mature B-cell neoplasms, was used: tube 1: non-staining (control tube); tube 2: CD19 (FITC), CD5 (PE), CD45 (PE-Cy5); tube 3: CD2 (FITC), CD23 (PE); tube 4: kappa (FITC), lambda (PE), CD19 (PE-Cy5); tube 5: FMC7 (FITC), CD79 (PE); tube 6: CD10 (FITC), CD11c (PE), CD38 (PE-Cy5); tube 7: CD103 (FITC), CD20 (PE); tube 8: IgM (FITC), CD200 (PE), CD19 (PE-Cy5); tube 9: CD8 (FITC), CD4 (PE), CD3 (PE-Cy5).[1] As traditionally recommended, the tubes containing antibodies against immunoglobulins were washed three times in PBS at room temperature. Of note, the clones of anti-kappa (FITC), anti-lambda (PE) and anti-CD19 (PE-Cy5) were G20-193 (isotype: mouse IgG1, k), JDC-12 (isotype: mouse IgG1, k) and HIB19 (isotype: mouse IgG1, k), respectively. All monoclonal antibodies were purchased from Becton Dickinson (San Jose, CA, USA), except anti-IgM (FITC), which were purchased from Dako (Carpinteria, CA, USA). A total of 10.000 cells per tube were acquired in a three-color COULTER EPICS XL-MCL flow cytometer (Beckman-Coulter).

Our first immunophenotypic analysis showed the presence of 75% of B-cells (CD19+) characterized by the co-expression of the CD5 antigen. The B-cells also exhibited positivity for the antigens CD11c, $CD20^{low}$, CD23, $CD45^{high}$, CD200, FMC7 and surface immunoglobulin IgM^{low} subtype. The B-cells were negative for the antigens CD2, CD3, CD4, CD8, CD10, CD25, CD38, CD79b and CD103. However, to our surprise, we were not able to detect either kappa or lambda expression on the surface of B-cells of tube 4 and, therefore, we were not initially able to confirm the light-chain restriction of them (Figure 1(a)).

We initially hypothesized to be dealing with: (a) a rare case of CLL characterized by the absence of surface light-chain immunoglobulin, that has previously been documented or, (b) the failure to detect the immunoglobulin light-chains as a consequence of anti-kappa and/or anti-lambda performance problems. However, the presence of surface IgM subtype heavy-chain on B-cells made us consider the alternative possibility that problems could be occurring with the set combination of reagents in the tube 4. Thus, we considered that the anti-CD19 (PE-Cy5) could be interfering with the binding of the anti-kappa (FITC) and/or the anti-lambda (PE) by means of SH. Alternatively, as a fourth hypothesis, we judged that fluorescence quenching could be the reason for the absence of kappa and/or lambda signals. So, we set about investigating the phenomenon: we initially performed another staining of tube 4, but now changing anti-CD19 (PE-Cy5) for anti-CD20 (PE-Cy5), clone 2H7, isotype mouse (C57BL/6 IgG2b), k. Yet, the phenomenon occurred anew and, thus, neither kappa nor lambda signals could be detected (data not shown).

We then tried an alternative approaching (the same blood specimen was used): we performed a new staining of tube 4, but this time adding sequentially the monoclonal antibodies: first, anti-kappa (FITC) and anti-lambda (PE) were added conjointly to the tube and, after 10 min of incubation, we finally added the anti-CD19 (PE-Cy5). The result is shown in Figure 1(b). The B-cells clearly exhibited $lambda^{low}$

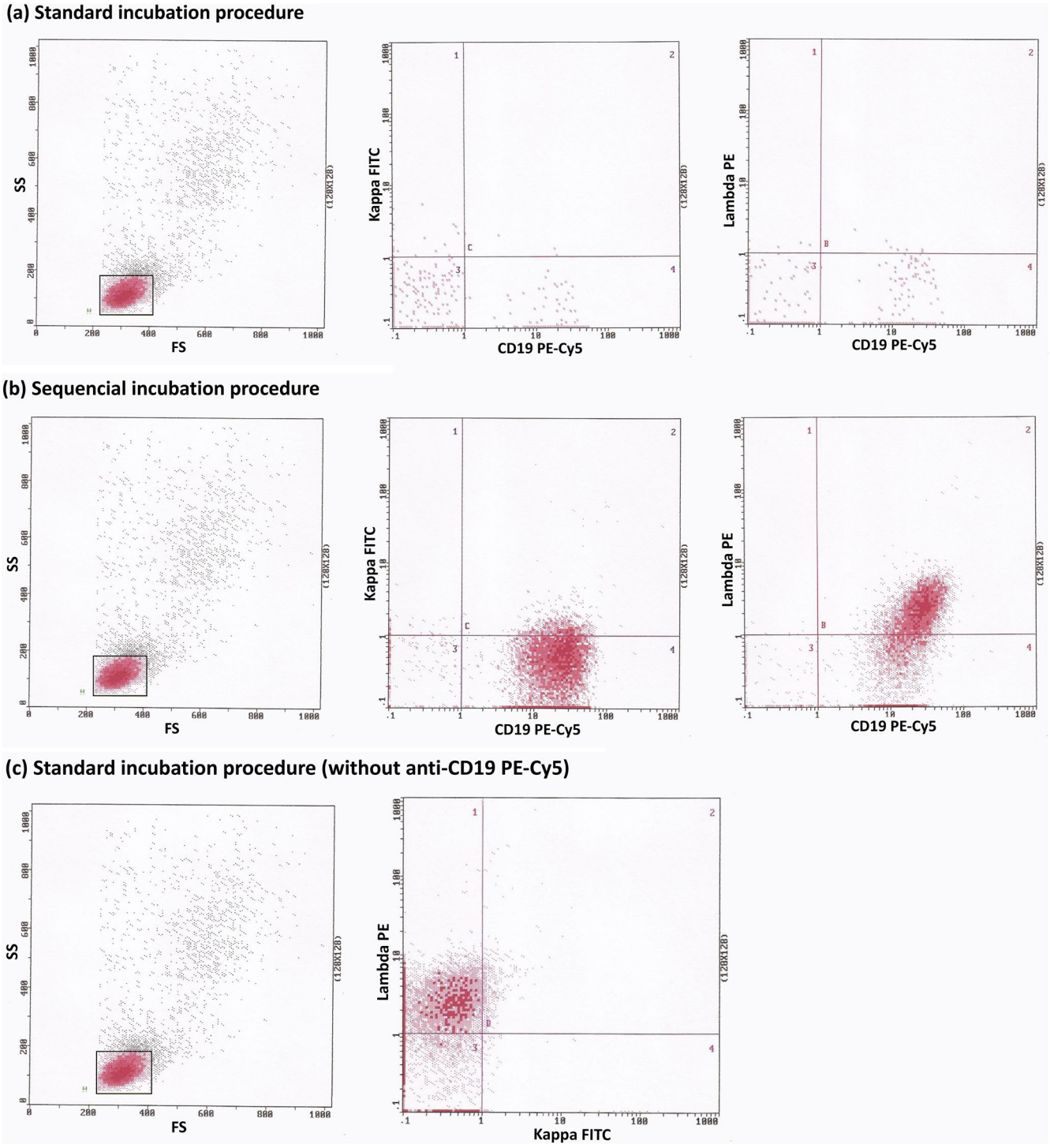

FIGURE 1 Flow cytometric immunophenotyping of peripheral blood sample. (a) Tube 4 (Procedure: standard incubation). Left dot-plot: Forward Scatter (FS) × Side Scatter (SS). The red gate delimits a population of lymphoid cells. Middle and right dot-plots: Kappa (FITC) x CD19 (PE-Cy5) and Lambda (PE) x CD19 (PE-Cy5), respectively. The plots show the presence of CLL B-cells (CD19+) and the absence of kappa and lambda signals because of the steric hindrance. (b) Tube 4 (Procedure: sequential incubation). Left dot-plot: Forward Scatter (FS) × Side Scatter (SS). The red gate delimits a population of lymphoid cells. Middle and right dot-plots: Kappa (FITC) x CD19 (PE-Cy5) and Lambda (PE) x CD19 (PE-Cy5), respectively. The plots show the presence of CLL B-cells (CD19+) characterized by lambda light-chain restriction. (c) Tube 4 (Procedure: standard incubation without the anti-CD19 PE-Cy5). Left dot-plot: Forward Scatter (FS) × Side Scatter (SS). The red gate delimits a population of lymphoid cells. Right dot-plot: Kappa (FITC) x Lambda (PE). The plot shows the presence of CLL B-cells (CD19+) characterized by lambda light-chain restriction [Color figure can be viewed at wileyonlinelibrary.com]

light-chain restriction. Therefore, our suspicion of SH was reinforced, to the detriment of the fluorescence quenching hypothesis, due to the fact that we would not expect that the sequential incubation procedure, which gave primacy of binding to anti-lambda (PE), would solve the problem of fluorescence quenching, because the possible energy transfer between PE and the tandem fluorochrome PE-Cy5

would still continue to occur. To confirm SH, we performed a new incubation, but this time without the anti-CD19 (PE-Cy5). The result is shown in Figure 1(C). Again, the B-cells exhibited lambda^{low} light-chain restriction.

Finally, to make the whole case more intriguing, we performed a minimal residual disease analysis of the bone marrow of the same patient, 17 months after the initial diagnosis. This time, tube 4 consisted of the set of monoclonal antibodies employed at diagnosis, namely, anti-kappa (FITC), anti-lambda (PE) and anti-CD19 (PE-Cy5), with the addition of anti-CD5 (APC). We found 0.4% of B-cells with the phenotype of CLL (CD5+, CD20^{low}, CD43+, CD79b-). The CLL-cells exhibited lambda^{low} light-chain restriction. This time, curiously, no SH was seen.

Steric hindrance is a rare phenomenon mainly associated with the reduction of fluorescence intensity which, in the context where the level of expression of an antigen has clinical significance, could lead to an erroneous interpretation of flow cytometry results. In extreme cases, like the one that our report portrays, more than a "mere" decrease in fluorescence intensity, there is a total absence of the fluorescence signal.

In our case, we suggest that a possible explanation for the SH phenomenon was related to the spatial proximity of CD19 protein to the transmembrane immunoglobulin molecule of the B-cell receptor (BCR). However, it remains to be explained the reason why we found only a single case of SH in our series of immunophenotypic studies. In fact, between 2009 and 2017, we employed our standard immunophenotypic panel, specifically for the analysis of suspected cases of mature B-cell neoplasms, 62 times[2]: in none of them the tube 4 showed the SH phenomenon. Additionally, in 101 times, the tube 4 was used as part of other immunophenotypic panels: again, we found no additional SH cases.

We do not have a complete explanation for the rarity of the phenomenon, even when the same combination of monoclonal antibodies is used so often, unless we assume that factors related to the distribution of the CD19 protein and the BCR in the membrane of B-cells of *particular* samples could contribute to SH. In any event, beyond the well-known influence of the molecular weights of the competing fluorochromes on the triggering of SH (with the combined use of heavy molecules, such as, for instance, PE and PE-Cy5, being more powerful to generate the phenomenon) (Table 1), the mechanisms involved in the emergence of SH are not plainly understood, being even possible that the degree of density of microvilli on cell surface could contribute to preventing the binding of the reagent involved (Wang et al., 2014). In this sense, other techniques could be of help to uncover the mechanism responsible to the appearance of SH. For example, Scanning Electron Microscopy – though be a technique not available for many centers around the world – could show the presence of artifacts on the surface of cells where the SH phenomenon has been documented. However, with the aim to clarify the molecular mechanisms that govern the interaction between antigen and antibody in SH, Atomic Force Microscopy could be more useful, whereas this technique allows the determination of the adhesion force, between antigen and antibody, as the parameter to be measured.[3]

TABLE 1 Fluorophores used in flow cytometry: molecular weight and excitation laser

Fluorophores[c]	Molecular weight (Daltons)	Excitation laser line (nm)[a]
Simple organic fluorophores		
FITC	389 Da	488 nm
Pacific blue™	406 Da	405 nm
Cascade blue	596 Da	405 nm or 407 nm
Violet Fluor 450	600 Da	405 nm
Texas red	625 Da	561 nm or 594 nm
Alexa Fluor® 488	643 Da	488 nm
DyLight 405	793 Da	405 nm
Alexa Fluor® 594	820 Da	561 nm or 594 nm
Red Fluor 910	900 Da	633 nm
DyLight 488	1.0 kDa	488 nm
DyLight 650	1.1 kDa	594 nm or 633 nm
Alexa Fluor® 660	1.1 kDa	633 nm
Spark blue™ 550	1.2 kDa	488 nm
Alexa Fluor® 647	1.3 kDa	633 nm or 640 nm
Alexa Fluor® 700	1.4 kDa	633 nm or 640 nm
Spark NIR™ 685	3.1 kDa	633 nm
Protein-based fluorophores		
PerCP	35 kDa	488 nm
PerCP-eFluor 710[b]	≅ 36–37 kDa	488 nm
PerCP-Cyanine5.5[b]	≅ 37 kDa	488 nm
APC	105 kDa	633 nm or 640 nm
AmCyan	108 kDa	405 nm
APC-Cyanine7[b]	109 kDa	633 nm or 640 nm
APC-fire™ 750[b]	110 kDa	633 nm or 640 nm
PE	240 kDa	488 nm or 532 nm or 561 nm
PE-Cyanine5[b]	≅ 242 kDa	488 nm or 532 nm or 561 nm
PE-eFluor 610[#]	≅ 242–245 kDa	488 nm or 532 nm or 561 nm
PE-Cyanine7[b]	245 kDa	488 nm or 532 nm or 561 nm
PE-dazzle™ 594[b]	245 kDa	488 nm or 532 nm or 561 nm
PE-fire™ 640[b]	260 kDa	561 nm
Organic polymers		
Brilliant violet 421™	60–80 kDa	405 nm
Brilliant violet 510™	60–80 kDa	405 nm
Brilliant violet 605™	60–80 kDa	405 nm
Brilliant violet 711™	60–80 kDa	405 nm
Kiravia Multimers		
KIRAVIA blue 520™	8 kDa	488 nm

[a]Excitation Laser Line: (i) 405 nm = violet laser; (ii) 407 nm = krypton laser; (iii) 488 nm = blue laser (argon); (iv) 532 nm = green diode laser; (v) 561 nm = yellow-green laser; (vi) 594 nm = red-orange laser; (vii) 633 nm = red laser (HeNe); (viii) 640 nm = red laser.

[b]Fluorophore:protein (F/P) ratio: The ratio of the PE, PerCP and APC molecules to small organic dyes (eFluor, Cyanine5, Cyanine5.5, Cyanine 7, Dazzle 594 and Fire 640) is often dependent on the conjugation process. Thus, it varies and can be "lot-specific" and "vendor-specific".

[c]Sources: Thermo Fisher Scientific (personal communication), Biolegend (personal communication) and https://www.biolegend.com/en-us/fluorophore-families and Cell Signaling Technology (personal communication).

Having said that, based on the results of our experiments performed to elucidate the staining inconsistences of tube 4, we can assert that SH is characteristically an asymmetrical phenomenon: when combined, a reagent "X" hampers a reagent "Y" to bind to its specific antigen "z", but the reagent "Y" not-necessarily hampers the reagent "X" to bind to its specific antigen "w". This contrasts with some staining problems related to the performance of monoclonal and polyclonal antibodies with specificity for the surface immunoglobulins light-chains, where the phenomenon has a symmetrical presentation: there is no fluorescence signal for both kappa and lambda light-chains.

In this train of thought, an equivalent experiment of sequential incubation was recently published and the presence of interference between the monoclonal antibodies, even when added at different times in the staining tube, suggested to the authors alternative mechanisms for the reduced fluorescence signal (De Vita et al., 2015). Of note, a related phenomenon of interference, fluorescence quenching, is also an asymmetrical phenomenon, but we believe that the experiment of sequential incubation that we performed could be used as simple method to help in the discrimination between SH and fluorescence quenching, without the use of complex experiments involving FRET (fluorescence-resonance energy transfer). FRET is possibly, although not exclusively, a mechanism of fluorescence quenching. FRET decreases the intensity of the donor fluorochrome and transfers the energy to an acceptor (De Vita et al., 2015).

In this sense, it is possible to speculate that, in our case, if fluorescence quenching was *ex-hypothesi* the very phenomenon underlying the absence of anti-lambda (PE) signal, the mechanism would be as follows: the fluorescence of the anti-lambda (PE) monoclonal antibody would be attenuated (or, as in our case, extinguished) by the anti-CD19 (PE-Cy5), which could absorb the anti-lambda (PE) derived energy (De Vita et al., 2015). But if this were true, the sequential incubation experiment performed by us would not restitute the anti-lambda (PE) signal. Thus, we sustain that the anti-lambda (PE) signal started to be seen because the mechanism underlying the absence of anti-lambda (PE) signal was SH, not fluorescence quenching. Accordingly, we suggest that the sequential incubation experiment we performed could be a suitable and easy test to discriminate between SH and fluorescence quenching in the practical of flow cytometry laboratories. Lastly, an alternative approach not tried by us, but that could eventually have solved the problem of SH as well, would be the exchange of PE-Cy5 for a fluorochrome of lower molecular weight (Table 1).

In the last decades, the number of fluorescent dyes available to be used in flow cytometry clinical and research studies has increased substantially. In fact, multiple lasers flow cytometers having two physical parameters (FSC and SSC) and 18 fluorescence detectors are relatively common nowadays. Instruments with more than 30 parameters, though of less common use, are also commercially available as, for instance, ID7000™ Spectral Cell Analyzer (more than 44 parameters; Sony Biotechnology), BD FACSymphony™ (50 parameters; Becton Dickinson) and Cytek® Aurora (67 parameters; Cytek Biosciences). Nevertheless, as it has been recently pointed out (De Vita et al., 2015), when creating reagent panels for immunophenotyping, the focus of attention is mainly on how to ensure consistency of reagents as well as color compensation issues, whereas the impact of a possible interference between monoclonal antibodies is generally not routinely tested. The problem is even more serious because, nowadays, most flow cytometry laboratories are relying on specialized softwares to manage color compensation, which can include over a dozen fluorochromes. Thus, in a nutshell, our report reinforces the necessity to look more carefully to the fluorochrome mutual interference issue, before abnormal levels of antigen expression can be asserted or even rare phenotypes of hematologic diseases can be considered and, consequently, incorrectly diagnosed in the landscape of polychromatic flow cytometry.

KEYWORDS

immunophenotyping, steric hindrance, quenching; surface immunoglobulin light-chain, flow cytometry.

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CONFLICT OF INTEREST

The author reports no conflict of interest.

AUTHOR CONTRIBUTIONS

D.M.M performed flow cytometry analysis, reviewed the case, reviewed the literature and wrote all the manuscript.

ENDNOTES

[1] Immunophenotypic panel (target cells): tube (1): non-staining lymphocytes; tube (2): mature B-lymphocytes and CD5+ B-lymphocytes; tube (3): T-lymphocytes and CD23+ B-lymphocytes; tube (4): mature B-lymphocytes; tube (5): B-lymphocytes; tube (6): germinal center B- lymphocytes, terminally differentiated B- lymphocytes and other subsets of B-lymphocytes; tube (7): T-lymphocytes and abnormal B-cells (hairy cell leukemia); tube (8): mature B-lymphocytes and other subset of B-lymphocytes; tube (9): subsets of T-lymphocytes and rare CD8+ CLL.
Of note: for a discussion about the uncommon inclusion of tube 9 (with only lineage T-cell markers) in an immunophenotypic panel specific for suspected cases of mature B-cell neoplasms, see: Matos DM. CD8 Antigen Expression in Chronic Lymphocytic Leukemia: Does it Have any Relevant Meaning? Cytometry Part B (Clinical Cytometry) 96B:96–98 (2019).

[2] From 2015 onwards, we started working with a BD FACSCalibur 4-color flow cytometer and, thus, we modified tube 4, in such a way as to add the monoclonal antibody anti-CD5 (APC) on it. Thus, tube 4: kappa

(FITC), lambda (PE), CD19 (PE-Cy5), CD5 (APC). Eventually, we customized the tube 4, changing CD19 (PE-Cy5) for CD19 (PerCP).

[3] The very preliminary idea would be to functionalize the tip of the Atomic Force Microscope with the antigen to be tested on the lymphocyte's membrane and, then, measure the adhesion force between antigen and antibody. Of note, to use Atomic Force Microscopy, the cells need to be attached on a solid surface (a solid surface coated with fibrin, for example). I am very grateful to Professor Nicole Jaffrezic-Renault (Emeritus Research Director in CNRS, Institute of Analytical Sciences, University of Lyon, France) for our brief, but very stimulating and rich, discussion about the possibilities of using Atomic Force Microscopy for clarifying some underlying mechanisms responsible for the phenomenon of SH.

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REFERENCES

De Vita, M., Catzola, V., Buzzonetti, A., Fossati, M., Battaglia, A., Zamai, L., & Fattorossi, A. (2015). Unexpected interference in cell surface staining by monoclonal antibodies to unrelated antigens. *Cytometry. Part B, Clinical Cytometry*, *88*(5), 352–354.

Meiyao Wang, Martin Misakian, Hua-Jun He, Peter Bajcsy, Fatima Abbasi, Jeffrey M Davis, Kenneth D Cole, Illarion V Turko, Lili Wang. Quantifying CD4 receptor protein in two human CD4+ lymphocyte preparations for quantitative flow cytometry. *Clinical Proteomics* 2014; *11*(1): 43.