



OPEN ACCESS

EDITED BY

Marco Rosati,
AstraZeneca, United Kingdom

REVIEWED BY

Naoki Sakurai,
Nihon University, Japan
Dominik von La Roche,
Ludwig Maximilian University of Munich,
Germany

*CORRESPONDENCE

Alessandra Ubiali
✉ alessandra.ubiali@unimi.it

RECEIVED 12 December 2025

REVISED 12 February 2026

ACCEPTED 06 March 2026

PUBLISHED 02 April 2026

CITATION

Ubiali A, Gariboldi EM, Stefanello D,
Ferrari R and Martini V (2026)
Fluorescence changes at flow
cytometric analysis of samples from
sentinel lymph nodes excised with
indocyanine green and methylene blue
guided mapping techniques.
Front. Vet. Sci. 13:1766355.
doi: 10.3389/fvets.2026.1766355

COPYRIGHT

© 2026 Ubiali, Gariboldi, Stefanello,
Ferrari and Martini. This is an
open-access article distributed under
the terms of the [Creative Commons
Attribution License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use,
distribution or reproduction in other
forums is permitted, provided the
original author(s) and the copyright
owner(s) are credited and that the
original publication in this journal is
cited, in accordance with accepted
academic practice. No use, distribution
or reproduction is permitted which does
not comply with these terms.

Fluorescence changes at flow cytometric analysis of samples from sentinel lymph nodes excised with indocyanine green and methylene blue guided mapping techniques

Alessandra Ubiali*, Elisa Maria Gariboldi, Damiano Stefanello, Roberta Ferrari and Valeria Martini

Dipartimento di Medicina Veterinaria e Scienze Animali, Università degli Studi di Milano, Lodi, Italy

Introduction: Flow cytometry (FC) has been used recently to assess percentages of infiltration by mast cells in sentinel lymph nodes (SLN) of dogs with mast cell tumors (MCT). SLN mapping often includes the use of different dyes such as methylene blue (MB) and indocyanine green (ICG), which might influence fluorescence assessment by FC. This study aimed to assess whether the color given by mapping dyes might affect the baseline fluorescence of SLN aspirates for FC.

Methods: Baseline fluorescence was calculated as Median Fluorescence Intensity (MFI) in 4 channels (FL1, FL2, FL3, FL4, respectively corresponding to 530/30 nm, 585/40 nm, >670 nm, 660/20 nm) with a cytometer equipped with two lasers (488 nm and 638 nm) with constant setting and compensation. SLN aspirates were suspended in RPMI medium. They were classified based on results of mapping techniques in 4 dye classes (blue/fluorescent, fluorescent/non-blue, blue/non-fluorescent, non-blue/non-fluorescent), and possible differences in MFI values among SLN dye classes were assessed.

Results: Thirty-five SLNs from 17 dogs were assessed. Considering dye classes, 13 were blue/fluorescent, 16 were fluorescent/non-blue, 4 were blue/non-fluorescent, 2 were non-blue/non-fluorescent. In all fluorescence channels, except FL1, MFI varied among SLN dye classes. Blue/non-fluorescent SLNs showed the highest fluorescence, followed by blue/fluorescent, fluorescent/non-blue, and non-blue/non-fluorescent.

Discussion: These results suggest that the use of dyes for SLN mapping may introduce a relevant bias when MFI is quantitatively assessed via FC.

KEYWORDS

flow cytometry, indocyanine green, median fluorescence intensity, methylene blue, sentinel lymph node

1 Introduction

Flow cytometry (FC) technique is based on lasers that emit light at specific wavelengths and hit sample cells in a liquid stream. Specific staining kits or fluorochrome-labelled antibodies can be added to the cellular suspension before analysis. Once excited by the laser light, the fluorochrome emits light at a different wavelength. That light is thereafter filtered to obtain a clearer

signal, recorded by specific detectors, and finally converted into numerical data by an electronic network. The higher the amount of fluorescent antibody binds to the cell, the higher the intensity of light at that specific wavelength will be produced. Finally, the numerical data obtained (Median Fluorescence Intensity, MFI) are proportional to the intensity of the light resulting from the interaction between the laser beam and the antibody-labelled cell, which in turn is proportional to the amount of protein present on the cell. Thus, besides assessing the relative prevalence of specific cellular subsets, FC plays a relevant role in assessing the degree of expression of specific molecules, enabling to move from a qualitative assessment to a quantitative one. This is commonly calculated as a ratio between the MFI obtained by antibody-labelled cells and the autofluorescence of the cells in an unstained sample (1, 2). Indeed, when cells are interrogated by the lasers, a minimal degree of light is captured by the detectors at different wavelengths even if cells were not pre-labelled with fluorescent substances, resulting in the so-called autofluorescence (3). FC has been recently proposed as a potential alternative for diagnosing nodal metastasis in dogs with mast cell tumor (MCT) (4, 5) and, even if histopathology remains the gold standard for assessing nodal metastasis in dogs, it seems plausible that FC will be further investigated in the context of sentinel lymph node (SLN) assessment in the future. To date, SLN mapping is a diffuse technique in surgical oncology of dogs, particularly for patients with MCT, and different mapping techniques have been assessed to correctly identify and extirpate the tumor-draining lymph nodes (6–10). Most of those techniques are the same of those commonly used also in human patients and include the use of specific tracers, such as Technetium-99 metastable (^{99m}Tc) for lymphoscintigraphy (8, 11–15), or dyes, such as methylene blue (MB) (8, 11) and indocyanine green (ICG) (9, 12, 14–18). In particular, ^{99m}Tc is invisible to the naked eye, while MB is detectable in the visible light spectrum (19, 20), and ICG emits in the near-infrared light spectrum (820 nm) once excited by 750–800 nm wavelength light (21, 22). Being injected peritumorally, those dyes follow the lymphatic route that connects MCT to the SLN, highlighting the latter within the sentinel lymphocentrum, ultimately resulting in an intraoperative guidance for the surgeon.

Aside from its well-recognized role in the investigation of hematological malignancies (23–25), in veterinary medicine, interest has recently extended to FC assessment of metastasis in SLN of dogs with MCT (4, 5). It seems quite straightforward that, when using FC on a sample obtained from an SLN, dyes like ICG and MB may interfere with the evaluation of autofluorescence.

Consequently, sampling an SLN that appears fluorescent or blue *in vivo* may lead to signal interference in FC, which is based on fluorescence detection.

This may occur specifically, if the emission spectrum of the dye overlaps with the fluorescence detection channels of the cytometer, or non-specifically, due to background emission within the spectral range of interest for FC (3, 26). Indeed, these molecules may exert a secondary optical effect on the detected fluorescence. Moreover, when two different dyes are used in the same patient, their interaction could potentially alter their spectral architecture, possibly leading to unexpected distortions in the emitted wavelengths.

Determining whether such alterations occur, and to what extent each fluorescence channel is affected, could provide valuable insights for the future evaluation of SLN in patients undergone to MB or ICG mapping procedures. Hence, this study aimed to assess the influence of those mapping dyes used either alone or in combination, on the autofluorescence at FC analysis of cells from *ex vivo* SLN aspirates of dogs with MCTs.

2 Methods

All samples included in this prospective study came from client-owned dogs bearing MCT that were subjected to sentinel lymphadenectomy after their owners signed a written informed consent, at the Veterinary Teaching Hospital, University of Milan, from September 2023 to December 2024. Since samples were taken *ex vivo* on excised lymph nodes, the study did not involve any additional procedure for tissue sampling above the planned surgical procedure; therefore, specific Ethical Committee approval to use leftover specimens for research purposes was not required (Ethical Committee decision 29 October 2012, renewed with protocol 02–2016, University of Milan).

Techniques used for mapping and detection of the SLN were: lymphoscintigraphy with ^{99m}Tc and near infrared fluorescence (NIRF) with ICG (9) with or without MB, lymphoscintigraphy with ^{99m}Tc with MB, near infrared fluorescence (NIRF) with ICG with or without MB (6, 8, 17, 27). Thus, SLN were qualitatively assessed as described in Gariboldi et al. (9) and defined as follows: blue/non-blue according to the direct visualization of the SLN and fluorescent/non-fluorescent based on intraoperative assessment using a specific portable handheld camera for NIRF detection (SPY-PHI, Stryker, MIDA, Tecnologia Medica S.p.a). SLNs that were non blue and positive only at lymphoscintigraphy assessment using a handheld intraoperative gamma-probe (HIGP–Crystal probe SG04; Crystal Photonic GmbH, Berlin, Germany), either because ICG was not used, or because NIRF-ICG assessment showed absence of fluorescence, were classified as non-blue/non-fluorescent.

Finally, SLNs were divided into four dye-classes based on mapping results: blue/fluorescent, fluorescent/non-blue, blue/non-fluorescent, and non-blue/non-fluorescent.

All extirpated SLNs were immediately sampled via fine needle aspiration (FNA) in the operating room, irrespective of the dye class assigned. The material was suspended in RPMI medium and processed for FC within 24 h from collection. Both the primary lesion and the extirpated SLN(s) were then subject to histopathological assessment for diagnostic and staging purposes.

For FC assessment, samples were processed according to the standard procedures of the laboratory, which have already been described in the literature (28). Briefly, an automated cell count was performed, and different sample volumes were placed in a tube containing a final count of 500,000 cells. Subsequently, 25 μL of a blocking solution containing 10% fetal bovine serum (FBS) and 0.2% sodium azide in RPMI was added. The tubes were incubated for 10 min, and then 1 mL of an RBC lysis buffer was added. Samples were centrifuged at 1500 rpm for 8 min; the supernatant was removed, and cell pellets were resuspended in 500 μL of PBS. Further tubes were prepared in the same way, and incubated with different antibody cocktails, for the purposes of another concomitant project. Acquisition was made with a flow cytometer (BriCyte E6, Mindray, Shenzhen, China) which was equipped with 2 lasers (488 nm and 638 nm) and had 4 fluorescence channels (FL1, FL2, FL3, FL4, respectively, corresponding to 530/30 nm, 585/40 nm, >670 nm, 660/20 nm). At the beginning of each laboratory session, a quality control check was performed with cytometer-specific controls (Rainbow Calibration Particles, Mindray). Instrument setting and compensation matrix were then kept constant. Specifically, the compensation matrix was generated using cells from nodal aspirates routinely processed in our laboratory for diagnostic purposes. All scattergrams were visually inspected during acquisition and analysis, and no evidence of over- or under-compensation was observed. Data were analyzed with specific software “MRflow”

(Mindray) by a single experienced operator (VM), who was blind to the mapping procedure used and to the dye class of each specific SLN. A gate was set to include only lymphoid cells and exclude platelets and debris. A second gate was set to exclude doublets. Finally, fluorescence data of unstained samples were recorded and reported as MFI for each fluorescence channel (Figure 1).

2.1 Statistical analyses

Descriptive statistics were calculated. Data from continuous variables were reported as median (1st and 3rd quartile), to ensure consistency and robustness of their presentation.

Statistical differences in MFI values of unstained samples among the four SLN dye classes were assessed with Generalized Estimating Equations (GEE) for gamma distribution with log link and sandwich standard errors. The choice of GEE was based on the observation that 9 dogs contributed to the study with multiple samples (from 2 to 5 samples each). Thus, considering the correlation among samples coming from the same patient, and the unequal cluster sizes, case ID was considered as a clustering unit for the analysis, with an exchangeable correlation structure. Models were fit for fluorescence channel.

All analyses were performed with SPSS v 29.0 for Windows, and significance was set at $p \leq 0.05$ for all tests.

3 Results

A total of 17 dogs were included. Based on tracers availability, in order to ensure a better identification, detection and removal of SLN, the mapping technique used was a combination of NIRF-ICG plus MB in 7 dogs, NIRF-ICG plus lymphoscintigraphy in 4, NIRF-ICG plus lymphoscintigraphy plus MB in 3, in 2 lymphoscintigraphy plus MB, and lastly, in one dog, NIRF-ICG only was used. A total of 35 SLNs were excised and included for FC evaluation. The subdivision in the 4 dye classes is reported in Table 1.

Overall, median MFI of unstained samples was 154 (range, 100–394) for FL1, 67 (range, 42–392) for FL2, 175 (range, 51–5278) for FL3, and 79 (range, 11.9–15,575) for FL4.

Median (1st and 3rd quartile) of samples for each dye class are reported in Table 1 and depicted in Figure 2. A significant difference in MFI values among SLN dye classes was detected for FL2 ($p = 0.002$),

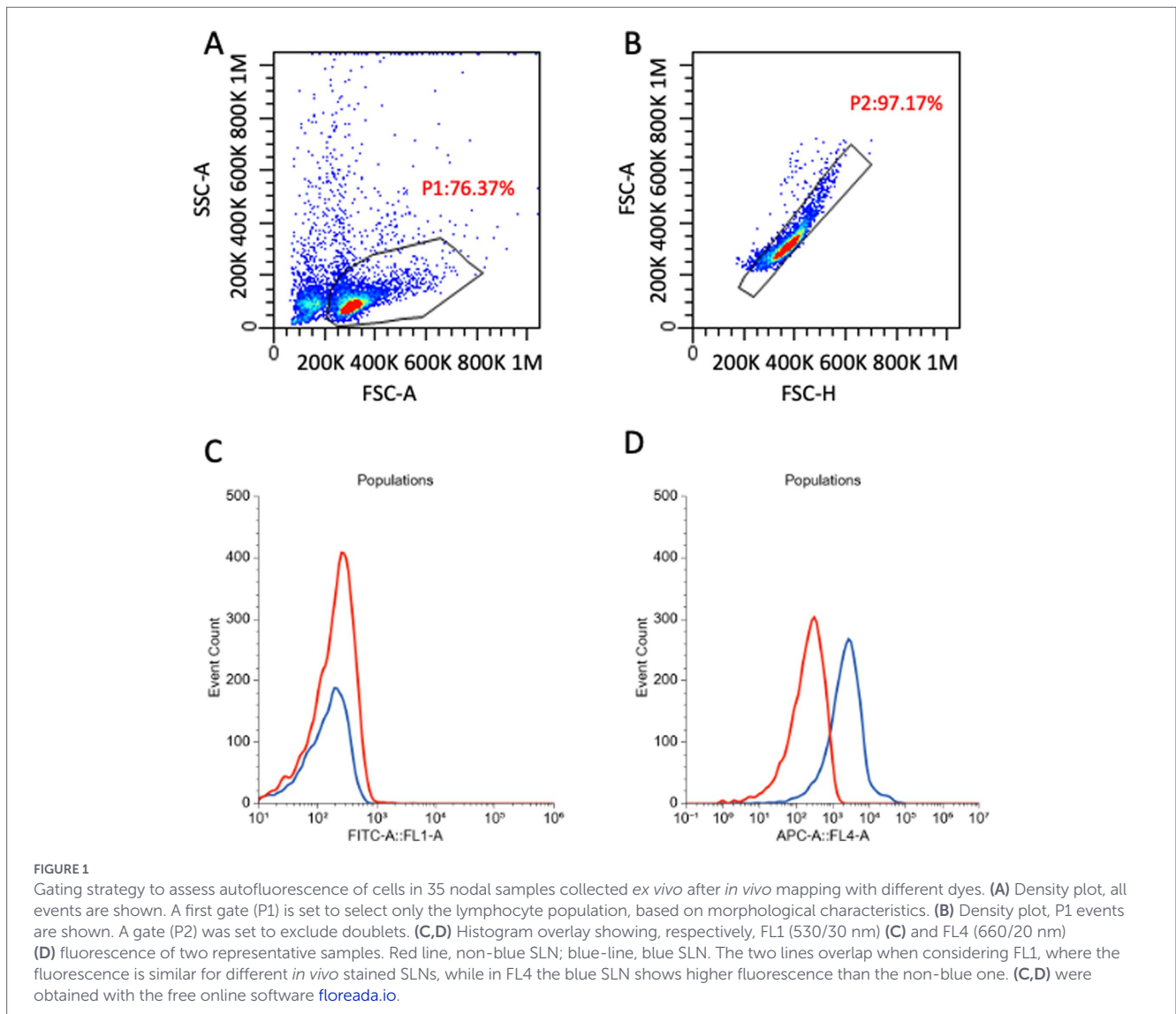


TABLE 1 MFI values of the four fluorescence channels based on SLN dye classes.

SLN dye class	n (%) total = 35	MFI fluorescence channel median (IQR)			
		FL1	FL2	FL3	FL4
Blue/fluorescent	13 (37%)	152.5 (122.0–215.0)	72.0 (56.5–128.0)	265.0 (96.5–686.0)	301.0 (59.0–751.5)
Fluorescent/non-blue	16 (46%)	156.5 (137.3–179.8)	63.5 (54.3–80.5)	119.5 (93.9–161.3)	40.0(23.5–97.0)
Blue/non-fluorescent	4 (11%)	245.5 (106.3–391.5)	196.5 (58.0–376.3)	1426.0 (411.8–4473.5)	2394.0 (805.3–12402.3)
Non-blue/non-fluorescent	2 (6%)	116.0; 335.0	5.0; 217.0	175.0; 400.5	50.5; 220.0

Notes: SLN, sentinel lymph node; MFI, median fluorescence intensity; IQR, interquartile range; FL, fluorescence channel.

FL3 ($p < 0.001$) and FL4 ($p < 0.001$), but not for FL1 ($p = 0.375$). In particular, blue/non-fluorescent SLN had the highest MFI values in all channels, blue/fluorescent SLN had intermediate values, whereas non-blue/non-fluorescent and non-blue/fluorescent SLN had overlapping, low values.

Despite differences in autofluorescence among classes, this variation did not impair lymphocyte population detection, which was always clearly defined (Supplementary Figure 1).

4 Discussion

In the present paper we reported a significant interference in the fluorescence intensity at three different wavelengths when either a single dye or a combination of MC and ICG was used for sentinel lymph node mapping and excision.

Each dye can emit light at specific wavelengths causing varying degrees of signal interference. Specifically, ICG is a fluorophore excited at 780–805 nm and emits at 820–840 nm (29). In our study, the lasers used operated at 488 and 638 nm. Thus, we did not expect significant interference from ICG. Conversely, MB, in addition to emitting within the visible light spectrum, has been reported to show an emission peak at 688 nm (30), which may partially overlap with the wavelengths detected in the present study (mainly FL3 and FL4, that are commonly used for PerCP, and APC/AF647 fluorochromes, respectively).

Our results demonstrated different prevalence of samples with high MFI values in FL2, FL3, and FL4 among blue/fluorescent, fluorescent/non-blue, blue/non-fluorescent, non-blue/non-fluorescent SLNs, leading to significant statistical differences. Conversely, higher overlap in MFI was found for FL1 channel, leading to lack of statistical differences (Table 1 and Figure 2). This result was partially expected, since the range of detection of FL1 (530/30 nm) lies far from the emission peaks of both MB and ICG. Still, minimal influence on autofluorescence even in the FL1 channel was present.

Regarding other fluorescence channels, blue/non-fluorescent SLNs showed the highest fluorescence, followed by blue/fluorescent

and fluorescent/non-blue and non-blue/non-fluorescent ones. This observation is consistent with the fact that MB is the only molecule reported to emit effectively within the wavelength range detected by our cytometer. Conversely, at FC assessment, ICG produced only a minor autofluorescence.

Interestingly, SLNs that were blue/fluorescent exhibited intermediate MFI values between blue/non-fluorescent and fluorescent/non-blue SLNs. This finding could be explained by possible alterations in the optical properties of MB and ICG when combined together, potentially due to molecular proximity or non-covalent interactions, anyway to the author knowledge no studies focused on this specific property.

Overall, our findings suggest that variations in the level of autofluorescence of the cells may introduce a relevant bias when MFI is quantitatively assessed in SLN from dogs that received either dye for SLN mapping. It could be speculated that nodal sampling before administration of any dye could help overcome this problem. We do not support such an approach, since the identification of the specific sentinel lymphocentrum and SLN within a lymphocentrum is only possible if using a mapping technique (8, 11, 17). Thus, sampling prior to the administration of any dye could lead to misidentification of the SLN. Awareness of the interference of each dye on FC results can help interpreting results from this technique, avoiding erroneous sampling of non-sentinel lymph nodes.

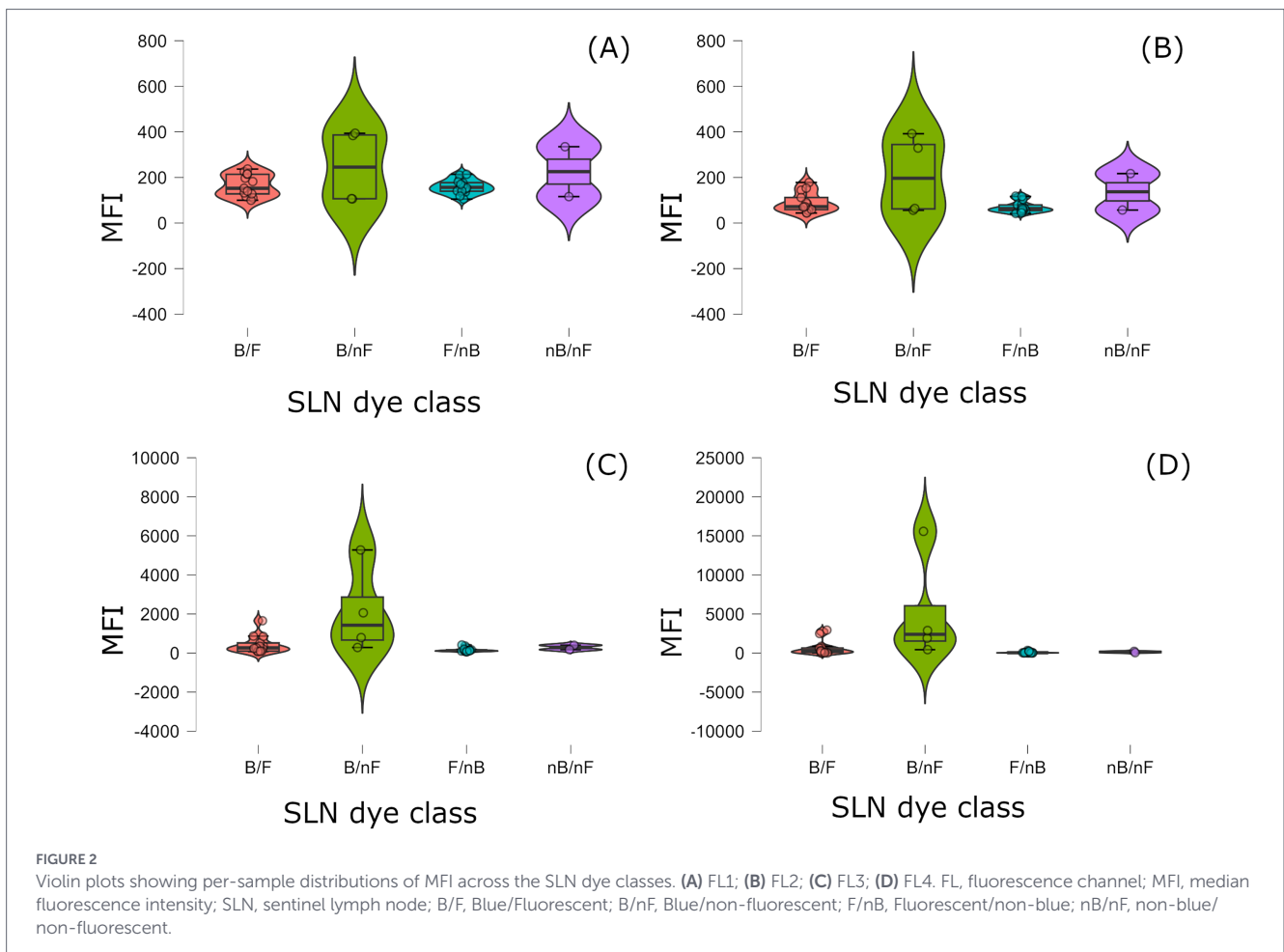
Furthermore, it is noteworthy to underlie that the influence on the autofluorescence of the cells did not prevent us to detect lymphocytes populations (Supplementary Figure 1), therefore the impact of intra-operative dyes has to be taken into account in a contest in which a quantitative assessment of the fluorescence of the sample is considered, and in that case, would be suggested to prefer SLNs detected by non-visible dyes such as lymphoscintigraphy with ^{99m}Tc , or to limit the analysis to the less affected fluorescence channel (FL1, 530/30 nm).

Considering that the injected dye typically follows the lymphatic flow to the lymph node, it is possible that part of it remains in the extracellular matrix while another portion is taken up by the cells. Therefore, performing multiple washes of the sample may help remove any unbound or freely diffusing dye, thereby reducing background fluorescence. This was not performed in the present study, since we aimed to mimic the standard operative procedures commonly used in veterinary FC facilities.

Anyway, the advantage of additional washing steps should be carefully evaluated and balanced with possible disadvantages, since it would result in additional sample handling, and, based on the authors' experience, it could cause cell loss and delay in sample acquisition and analysis.

The main limitations of the present study are related to its exploratory nature, mainly regarding the number of samples collected for each mapping technique and the inclusion of SLNs extirpated with different combinations of them. Most importantly, only 2 non-blue/non-fluorescent samples were included. This could have impacted on the statistical results of our study. Still, those two samples represented the true paragon of all other dye classes, since their MFI was linked to cellular autofluorescence, without uptake of any kind of dye.

In particular, either methylene blue or NIRF-ICG are often used in combination of other techniques as they help the surgeon to visualize SLN during extirpation, showing very good performances, with a really low number of SLN without blue or fluorescence uptake reported to date (9, 27, 31). The low number of non-fluorescent/non-blue SLN in the present study clearly reflects this tendency, but created an important limitation leading to a low number of samples for this category. In the future, to confirm this data, more samples have to be included. In



particular, expansion of the non-blue/non-fluorescent and blue-only groups would be necessary to strengthen statistical comparisons. Since sampling was performed in a clinical setting, and although all patients received the same peritumoral dose of tracer, the actual concentration, dilution, and distribution of dyes were beyond our control and could not be measured.

Consequently, part of our results may have been influenced by patient-specific characteristics of drainage, leading to different uptake of the dyes by SLNs overall from one dog to another. Anyway, this should have been partially prevented by use of GEE test rather than Kruskal-Wallis one, which would not have kept into consideration possible correlation among samples. Despite this, further variables that were not considered in the present study could possibly have biased the results, including timing from dye injection to sampling and processing for FC, as well as breed peculiarities or differences among anatomical location of the lymphocentrum involved, tumor cells burden and type of cells sampled (32–34), cell viability (35–37), and others. Those same variables could have led to the high intra-dye class variability observed in the present study. All of them need to be addressed in future studies.

5 Conclusion

ICG and MB, when used for SLN mapping, may influence the baseline fluorescence of cells in nodal aspirates during FC analysis. Specifically, MB has a stronger influence than ICG or their

combination, with FL2, FL3 and FL4 channels being more affected than FL1. This potential interference should be taken into account when performing quantitative fluorescence assessments on SLN samples obtained with mapping with MB and/or ICG in future studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study did not involve any additional procedure for tissue sampling above the planned surgical procedure; therefore, specific Ethical Committee approval to use leftover specimens for research purposes was not required (Ethical Committee decision 29 October 2012, renewed with protocol 02–2016, University of Milan). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AU: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing. EMG: Resources, Writing – review & editing. DS: Resources, Writing – review & editing. RF: Resources, Writing – review & editing. VM: Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Writing – review & editing.

Funding

The author(s) declared that financial support was received for this work and/or its publication. The authors acknowledge the support of the APC central fund of the University of Milan.

Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declared that Generative AI was not used in the creation of this manuscript.

References

- Advani AS, Rodriguez C, Jin T, Jawde RA, Saber W, Baz R, et al. Increased C-kit intensity is a poor prognostic factor for progression-free and overall survival in patients with newly diagnosed AML. *Leuk Res.* (2008) 32:913–8. doi: 10.1016/j.leukres.2007.08.019
- Gelain ME, Martini V, Giantin M, Aricò A, Poggi A, Aresu L, et al. CD44 in canine leukemia: analysis of mRNA and protein expression in peripheral blood. *Vet Immunol Immunopathol.* (2014) 159:91–6. doi: 10.1016/j.vetimm.2014.02.008
- Hulspas R, O'Gorman MR, Wood BL, Gratama JW, Sutherland DR. Considerations for the control of background fluorescence in clinical flow cytometry. *Cytometry B Clin Cytom.* (2009) 76B:355–64. doi: 10.1002/cyto.b.20485
- Chalfon C, Sabattini S, Riondato F, Iamone G, Renzi A, Ciammaichella L, et al. Prognostic impact of mast cell infiltration detected by flow cytometry on excised sentinel lymph nodes in dogs with newly diagnosed mast cell tumours. *Vet Comp Oncol.* (2025) 23:629–41. doi: 10.1111/vco.70018
- Iamone G, Chalfon C, Marconato L, Miniscalco B, Sabattini S, Agnoli C, et al. Flow cytometry for the detection and quantification of mast cells in lymph nodes: a prospective study in 64 dogs with mast cell tumour. *Vet Comp Oncol.* (2025) 23:1–9. doi: 10.1111/vco.13019
- Alvarez-Sanchez A, Townsend KL, Newsom L, Milovancev M, Gorman E, Russell DS. Comparison of indirect computed tomographic lymphography and near-infrared fluorescence sentinel lymph node mapping for integumentary canine mast cell tumors. *Vet Surg.* (2023) 52:416–27. doi: 10.1111/vsu.13929
- Chiti LE, Beer P, Ohlerth SM, Hartnack S, Nolf MC. SHINE – validation of near infrared fluorescence lymphography against lymphoscintigraphy for sentinel lymph node biopsy in dogs with mast cell tumours. *Vet Comp Oncol.* (2025) 23:320–9. doi: 10.1111/vco.13058
- Gariboldi EM, Ubiali A, Chiti LE, Ferrari R, De Zani D, Zani DD, et al. Evaluation of surgical aid of methylene blue in addition to intraoperative gamma probe for sentinel lymph node extirpation in 116 canine mast cell tumors (2017–2022). *Animals.* (2023) 13:1854. doi: 10.3390/ani13111854
- Gariboldi EM, Ubiali A, Boracchi P, Luconi E, Ferrari R, Auletta L, et al. Inpatient application of lymphoscintigraphy and near-infrared fluorescence in canine and feline

sentinel lymph node mapping and removal. *BMC Vet Res.* (2025) 21:590. doi: 10.1186/s12917-025-05013-2

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2026.1766355/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Example of a sample from a blue SLN that was stained with CD21-AF647 and CD45-FITC (A), CD5-PerCP-EF710 and CD45-FITC (B), CD4-FITC, and CD8-PE (C) antibodies to assess lymphocytes subpopulations. The populations are clearly identifiable and not affected by the baseline fluorescence due to the *in vivo* staining of the SLN.

- Stefanello D, Gariboldi EM, Boracchi P, Ferrari R, Ubiali A, De Zani D, et al. Weishaar's classification system for nodal metastasis in sentinel lymph nodes: clinical outcome in 94 dogs with mast cell tumor. *J Vet Intern Med.* (2024) 38:1675–85. doi: 10.1111/jvim.16997
- Ferrari R, Chiti LE, Manfredi M, Ravasio G, De Zani D, Zani DD, et al. Biopsy of sentinel lymph nodes after injection of methylene blue and lymphoscintigraphic guidance in 30 dogs with mast cell tumors. *Vet Surg.* (2020) 49:1099–108. doi: 10.1111/vsu.13483
- Kedrzycki MS, Leiloglou M, Ashrafian H, Jiwa N, Thiruchelvam PTR, Elson DS, et al. Meta-analysis comparing fluorescence imaging with radioisotope and blue dye-guided sentinel node identification for breast cancer surgery. *Ann Surg Oncol.* (2021) 28:3738–48. doi: 10.1245/s10434-020-09288-7
- Manfredi M, De Zani D, Chiti LE, Ferrari R, Stefanello D, Giudice C, et al. Preoperative planar lymphoscintigraphy allows for sentinel lymph node detection in 51 dogs improving staging accuracy: feasibility and pitfalls. *Vet Radiol Ultrasound.* (2021) 62:602–9. doi: 10.1111/vru.12995
- Somashekhar SP, Kumar CR, Ashwin KR, Zaveri SS, Jampani A, Ramya Y, et al. Can low-cost indo cyanine green fluorescence technique for sentinel lymph node biopsy replace dual dye (radio-colloid and blue dye) technique in early breast cancer: a prospective two-arm comparative study. *Clin Breast Cancer.* (2020) 20:e576–83. doi: 10.1016/j.clbc.2020.03.013
- Stoffels I, Dissemont J, Pöppel T, Schadendorf D, Klode J. Intraoperative fluorescence imaging for sentinel lymph node detection: prospective clinical trial to compare the usefulness of indocyanine green vs technetium Tc 99m for identification of sentinel lymph nodes. *JAMA Surg.* (2015) 150:617–23. doi: 10.1001/jamasurg.2014.3502
- Bargon CA, Huibers A, Young-Afat DA, Jansen BAM, Borel-Rinkes IHM, Lavalaye J, et al. Sentinel lymph node mapping in breast Cancer patients through fluorescent imaging using Indocyanine green: the INFLUENCE trial. *Ann Surg.* (2022) 276:913–20. doi: 10.1097/SLA.0000000000005633
- Beer P, Chiti LE, Nolf MC. The role of sentinel node mapping and lymphadenectomies in veterinary surgical oncology. *Lymphatics.* (2023) 1:2–18. doi: 10.3390/lymphatics1010002

18. da Silva Sá R, Von Ah Rodrigues RF, Bugalho LA, da Silva SU, Pinto Nazário AC. Evaluation of the efficacy of using indocyanine green associated with fluorescence in sentinel lymph node biopsy. *PLoS One*. (2023) 18:e0273886. doi: 10.1371/journal.pone.0273886
19. Barnes TG, Hompes R, Birks J, Mortensen NJ, Jones O, Lindsey I, et al. Methylene blue fluorescence of the ureter during colorectal surgery. *Surg Endosc*. (2018) 32:4036–43. doi: 10.1007/s00464-018-6219-8
20. Ginimuge PR, Jyothi SD. Methylene blue: revisited. *J Anaesthesiol Clin Pharmacol*. (2010) 26:517–20. doi: 10.4103/0970-9185.74599
21. Alander JT, Kaartinen I, Laakso A, Pätälä T, Spillmann T, Tuchin VV, et al. A review of indocyanine green fluorescent imaging in surgery. *Int J Biomed Imaging*. (2012) 2012:940585. doi: 10.1155/2012/940585
22. Mordon S, Devoisselle JM, Soulie-Begu S, Desmettre T. Indocyanine green: physico-chemical factors affecting its fluorescence *in vivo*. *Microvasc Res*. (1998) 55:146–52. doi: 10.1006/mvres.1998.2068
23. Evans SJM. Flow cytometry in veterinary practice. *Vet Clin North Am Small Anim Pract*. (2023) 53:89–100. doi: 10.1016/j.cvsm.2022.07.008
24. Marconato L, Polton GA, Sabattini S, Dacasto M, Garden OA, Grant I, et al. Conformity and controversies in the diagnosis, staging and follow-up evaluation of canine nodal lymphoma: a systematic review of the last 15 years of published literature. *Vet Comp Oncol*. (2017) 15:1029–40. doi: 10.1111/vco.12244
25. Martini V, Poggi A, Riondato F, Gelain ME, Aresu L, Comazzi S. Flow-cytometric detection of phenotypic aberrancies in canine small clear cell lymphoma. *Vet Comp Oncol*. (2015) 13:281–7. doi: 10.1111/vco.12043
26. Roederer M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry*. (2001) 45:194–205. doi: 10.1002/1097-0320(20011101)45:3<194::aid-cyto1163>3.0.co;2-c
27. Wan J, Oblak ML, Ram A, Singh A, Nykamp S. Determining agreement between preoperative computed tomography lymphography and indocyanine green near infrared fluorescence intraoperative imaging for sentinel lymph node mapping in dogs with oral tumours. *Vet Comp Oncol*. (2021) 19:295–303. doi: 10.1111/vco.12675
28. Riondato F, Poggi A, Miniscalco B, Sini F, Marconato L, Martini V. Flow cytometric features of B- and T-lymphocytes in reactive lymph nodes compared to their neoplastic counterparts in dogs. *Vet Sci*. (2023) 10:374. doi: 10.3390/vetsci10060374
29. Mytych W, Bartusik-Aebischer D, Aebischer D. The medical basis for the photoluminescence of indocyanine green. *Molecules*. (2025) 30:888. doi: 10.3390/molecules30040888
30. Cwalinski T, Polom W, Marano L, Roviello G, D'Angelo A, Cwalina N, et al. Methylene blue – current knowledge, fluorescent properties, and its future use. *J Clin Med*. (2020) 9:3538. doi: 10.3390/jcm9113538
31. Griffin MA, Flesner BK, Worley DR, Holt DE, Gill N, Ghanian A, et al. Incorporation of pre- and intra-operative sentinel lymph node mapping techniques in dogs with apocrine gland anal sac adenocarcinoma. *Vet Oncol*. (2024) 1. doi: 10.1186/s44356-024-00005-0
32. Baumann Z, Wiethe C, Vecchi CM, Richina V, Lopes T, Bentires-Alj M. Optimized full-spectrum flow cytometry panel for deep immunophenotyping of murine lungs. *Cell rep methods*. (2024) 4:100885. doi: 10.1016/j.crmeth.2024.100885
33. Bourdely P, Petti L, Khou S, Meghraoui-Kheddar A, Elaldi R, Cazareth J, et al. Autofluorescence identifies highly phagocytic tissue-resident macrophages in mouse and human skin and cutaneous squamous cell carcinoma. *Front Immunol*. (2022) 13:903069. doi: 10.3389/fimmu.2022.903069
34. Miranda-Lorenzo I, Dorado J, Lonardo E, Alcalá S, Serrano AG, Clausell-Tormos J, et al. Intracellular autofluorescence: a biomarker for epithelial cancer stem cells. *Nat Methods*. (2014) 11:1161–9. doi: 10.1038/nmeth.3112
35. Kozlova AA, Verkhovskii RA, Ermakov AV, Bratashov DN. Changes in autofluorescence level of live and dead cells for mouse cell lines. *J Fluoresc*. (2020) 30:1483–9. doi: 10.1007/s10895-020-02611-1
36. Surre J, Saint-Ruf C, Collin V, Orenga S, Ramjeet M, Matic I. Strong increase in the autofluorescence of cells signals struggle for survival. *Sci Rep*. (2018) 8:12088. doi: 10.1038/s41598-018-30623-2
37. Zamai L, Bareggi R, Santavenere E, Vitale M. Subtraction of autofluorescent dead cells from the lymphocyte flow cytometric binding assay. *Cytometry*. (1993) 14:951–4. doi: 10.1002/cyto.990140815