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COMMENTARY

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Expanding the use of flow cytometry in semen analysis: The rise of flow spermetry

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Although flow cytometry is not as popular in spermatology as in other areas of biology and medicine, is increasingly being used in this field [1, 2]. Spermatozoa, similarly, to blood cells, are individual cells suspended in a liquid (seminal plasma in spermatozoa) and are thus well suited for flow cytometry analysis. While flow cytometry has been used for a long time in spermatology (with earlier publications dating back to the 80s of the last century), most applications are still simple combinations of 1 or two probes and two colors. However, the complexity of flow cytometry analysis is increasing in the field of spermatology, as evidenced by Umair et al. [3]. The authors used a multiparametric approach to measure the production of reactive oxygen species (ROS), mitochondrial function, changes in Ca^{2+} concentration, and plasma membrane fluidity in viable acrosome intact spermatozoa. The authors concluded that prolonged storage of stallion spermatozoa at 5°C resulted in disturbed Ca^{2+} homeostasis and increased plasma membrane fluidity, demonstrating the power of flow cytometry disclosing the mechanisms of sperm aging during conservation. This study underlines the utility of flow cytometry in studying the biology of spermatozoa and its potential to rapidly translate the information gathered into clinical settings. Other recent papers published also demonstrate the utility of flow cytometry in clinical sperm analysis [4], and the study of the biology of the spermatozoa [5, 6].

One noteworthy aspect of the study by Umair et al. [3] is that flow cytometry generates vast information. The traditional approach to visualize the data used in spermatology—that is, dot plots and histograms—may obscure important information on the functional characteristics of the sample. Our laboratory has a 6-laser flow cytometry instrument that allows us to analyze multiple parameters in a single assay. Given the growing complexity of the flow cytometry assays applied to spermatology, the next logical step is using techniques for improved visualization and data analysis. Since 5 or more color experiments can be easily performed using a 6-laser instrument, the increased complexity of multiparametric assays requires changes in the data management and analysis. Most importantly post-acquisition analysis is crucial. Data should be collected and stored uncompensated and compensation must be done and computed when data is analyzed [7]. The appropriate way to set correct compensation is to rely on values computed by software from appropriately set control samples. [7]. Currently available software like FlowJo™ or Cytobank™ can compute and apply compensation, and in addition facilitate and enable visualization of all the events using one dimensional transformation of raw data to logicle, bi-exponential or arcsinh scales, in the fluorescence channels. This transformation voids the stacking of events against the axes of the traditional logarithmic scales, shifting fluorescence to 0 and negative values.

Another critical aspect to consider in the multiparametric analysis of the spermatozoa is the correct definition of boundaries. The first step to differentiate a dully stained sperm from

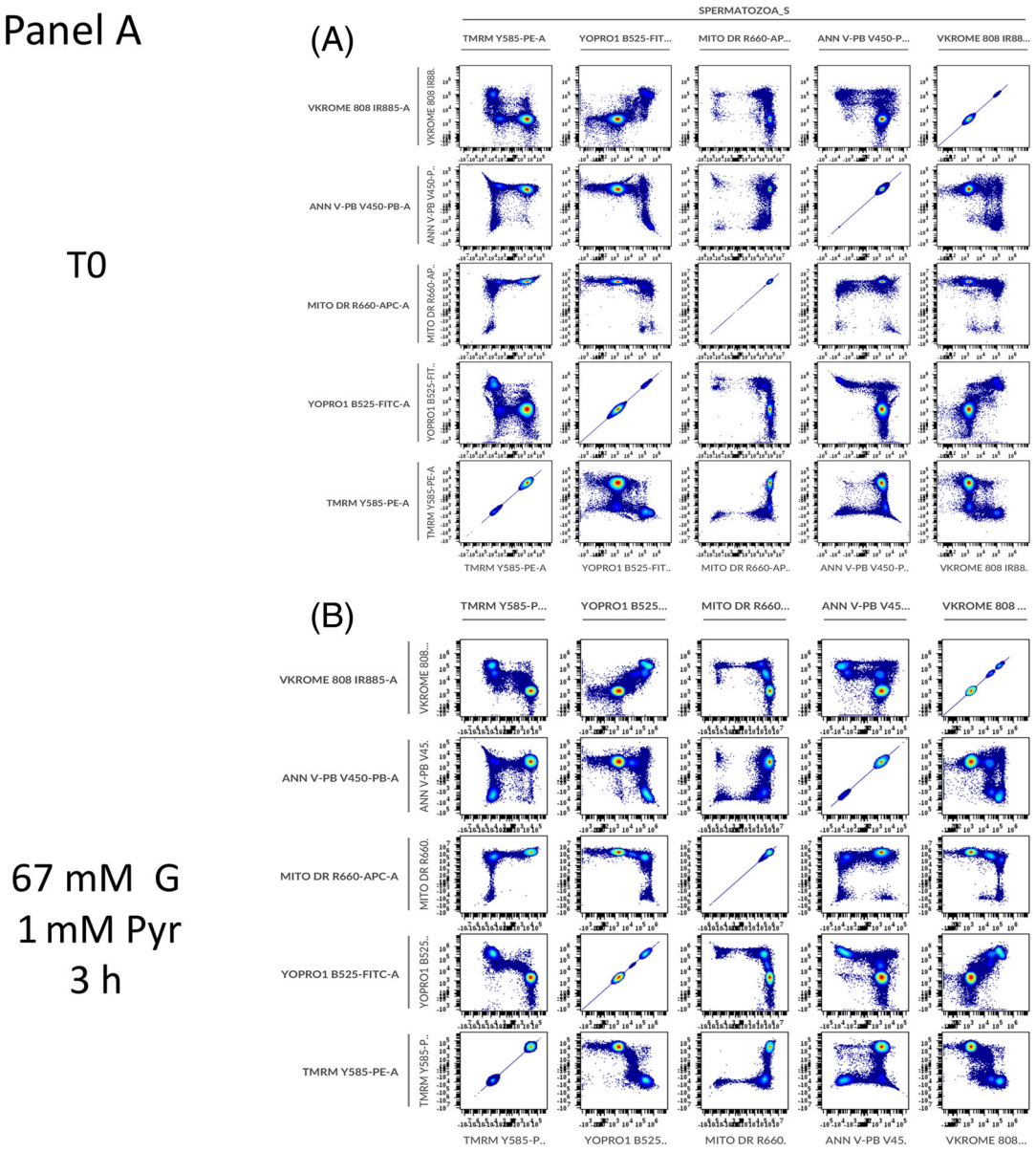
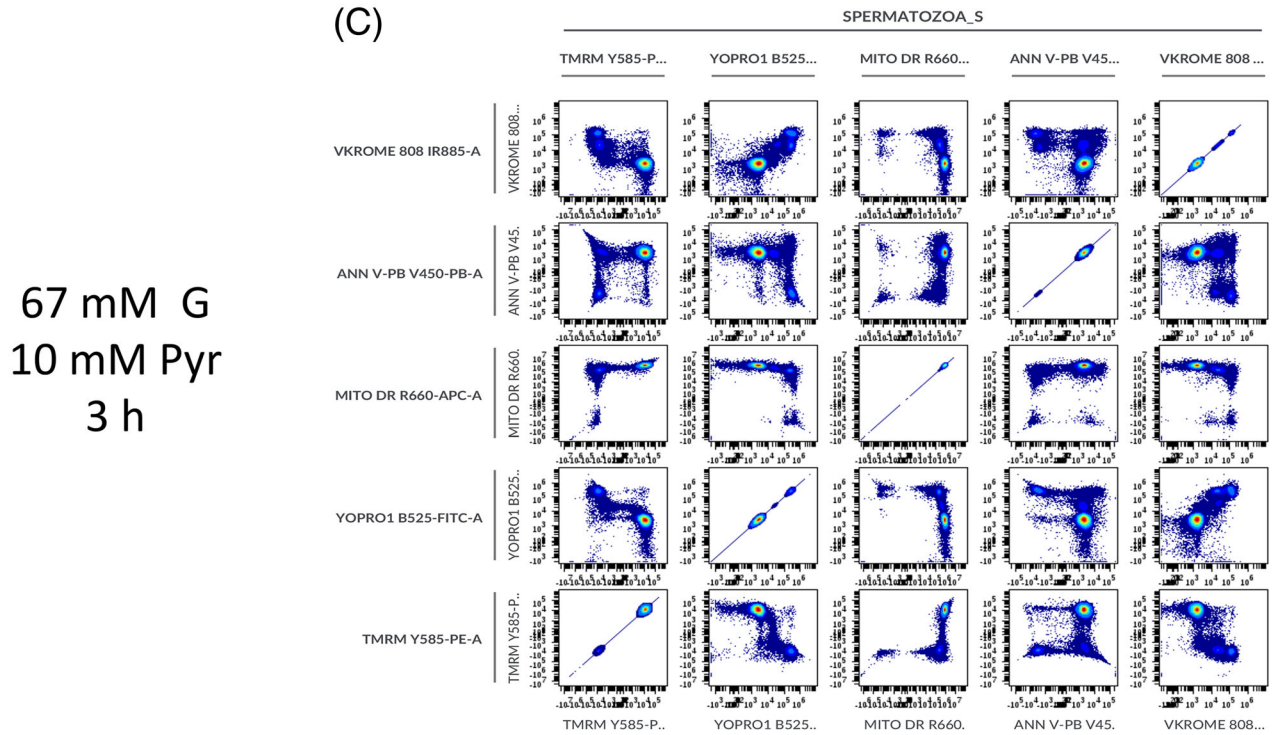


FIGURE 1 Example of how the use of artificial intelligence (self-learning) can improve the visualization and interpretation of data in a multiparametric analysis of spermatozoa. In this experiment, a five-color panel is used to evaluate viability, apoptosis, mitochondrial mass, and mitochondrial membrane potential. For this, the stallion spermatozoa are stained with Viakrome 808 (Ex 854 nm/Em 878 nm), YoPro-1 (Em 491 nm/Em 509), Annexin-V (Ex 410/Em 455 nm), Mitotracker deep red (Ex 644 nm/Em 655 nm) and (TMRM Ex 548 nm/Em 574 nm). Controls consisted of unstained and single-stained samples to establish compensation and boundaries. Data were analyzed in Cytobank v 10.1 (Beckman Coulter, Brea, CA) using PeacoQC [12] to automatically identify and remove anomalous events, and automatic compensation. Using a traditional approach, gates were manually created and pairwise dot plots were generated. As seen in this figure, panel A, for each experimental condition, 20 dot plots are generated, 60 panels total. This high number of panels difficult quick visualization of the changes amongst experimental conditions. Self-learning algorithms can improve the visualization and analysis of the data, in this case, the gating is done automatically by the algorithm reducing subjectivity. In this figure 1, panel 2, the t-SNE maps generated (D; 1–3) clearly show that the three conditions significantly impact the map generated. Both the number and size of the islands differ amongst groups; we gated each biggest island in each condition and investigated the functional phenotype of the spermatozoa (E and F). It can be seen that the percentages of live non-apoptotic spermatozoa (both Viakrome 808 and YoPro-1 negative; E-2) dropped after 3 h of incubation, as did the mitochondrial membrane potential (F-2) in spermatozoa incubated in 67 mM Glucose, 1 mM pyruvate, while spermatozoa incubated in a media containing 67 mM glucose and 10 mM pyruvate maintained initial values after 3 h of incubation. An interesting and easily visualizable finding is the striking difference between the mitochondrial mass and the mitochondrial membrane potential. In (G), t-SNE dop plot maps show each color used in the Z channel. As seen in * and # marked plots, not all the mitochondria (#) show high mitochondrial membrane potential (*). This aspect may be of great value for the study of mitochondrial dynamics in spermatozoa. [Color figure can be viewed at wileyonlinelibrary.com]

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A



Panel 2

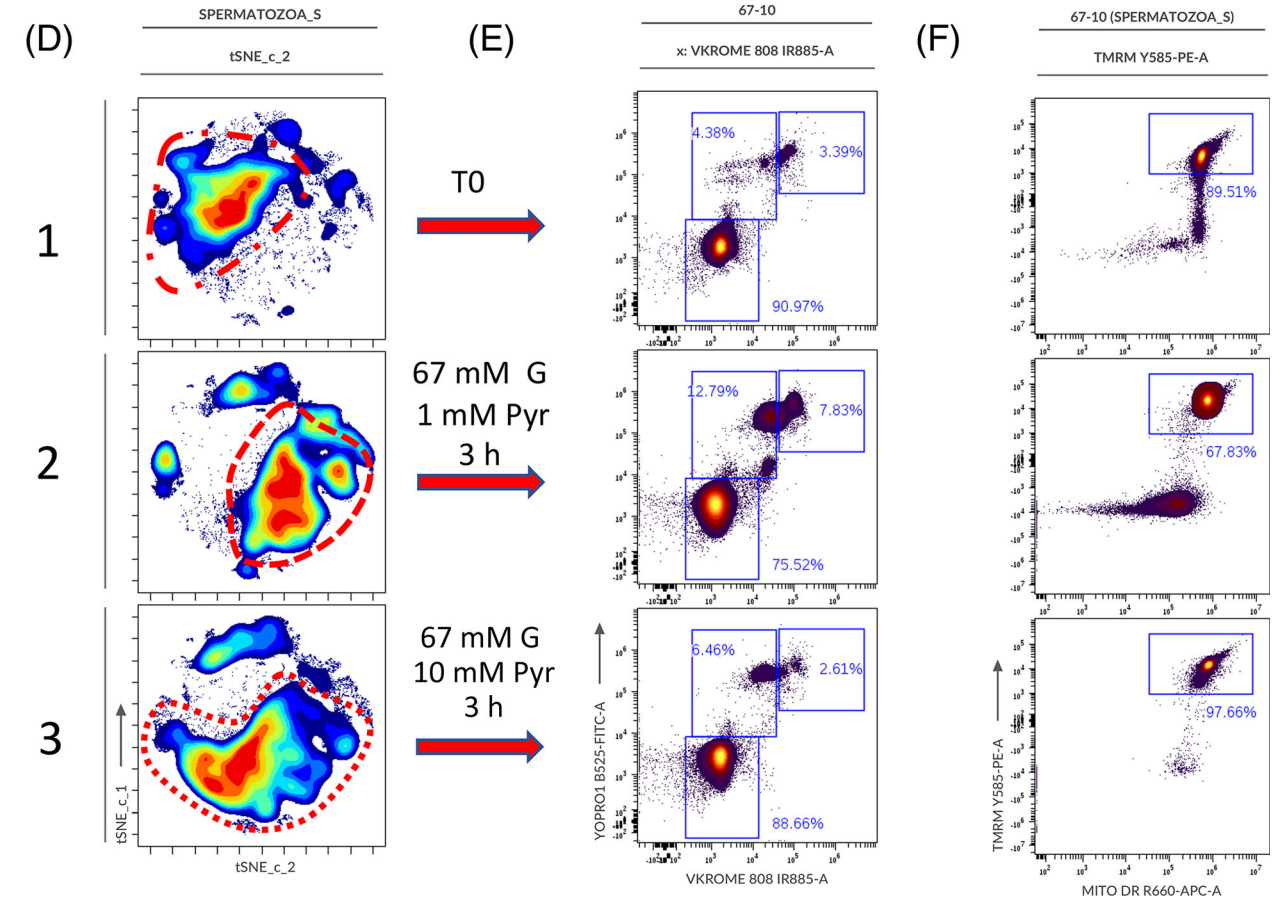


FIGURE 1 (Continued)

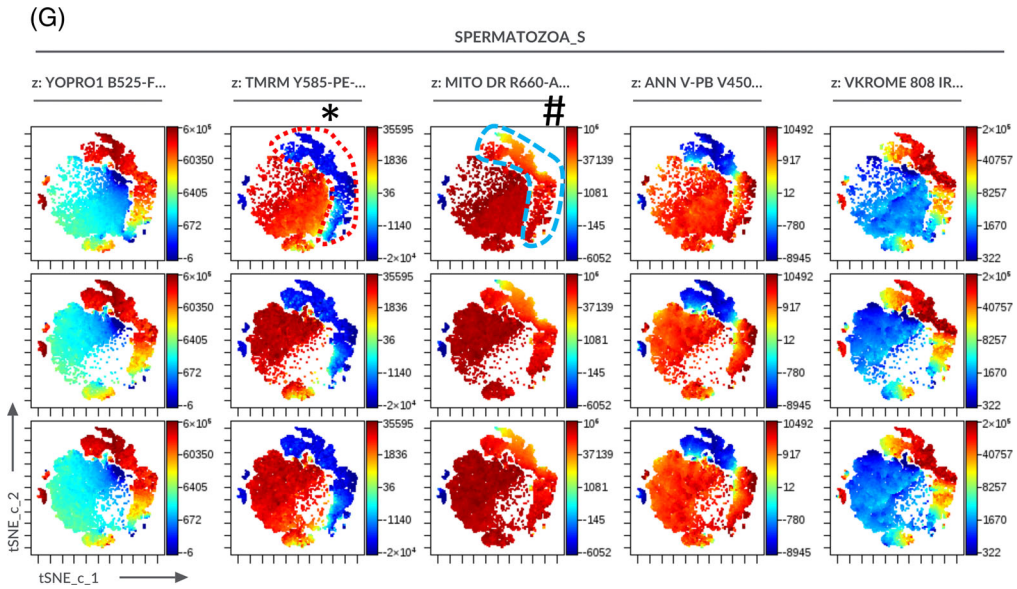


FIGURE 1 (Continued)

an unstained spermatozoon is to have unstained controls to determine the positivity boundary. However, the best way to determine the thresholds is the use of fluorescence minus one controls (FMO). In an FMO control set, all the probes to be used in a multicolor experiment are used, except the one for which the threshold is to be determined. The data from the FMO controls are collected, compensated, and displayed like the full stained population of spermatozoa. The background fluorescence for each subset is demonstrated by the upper boundary of the subset in the FMO control. Additionally, computational flow cytometry can improve the reproducibility of the analysis because these techniques are based on sound mathematical principles to define cluster boundaries, and the similarities between cells are assessed in all markers simultaneously [8]. Data visualization is another important aspect of the multiparametric analysis of spermatozoa. This aspect can be clearly observed in the following example of a five-color experiment (Figure 1). This experiment uses three probes to study the status of the sperm membrane; Viakrome 808 for viability, YOPRO-1 for membrane permeability, and Annexin-V, then also uses two probes to investigate sperm mitochondria, Mitotracker deep Red, to visualize changes in the mitochondrial mass, and TMRM to investigate the mitochondrial membrane potential. If data are analyzed using manual gating and visualized using the traditional approach of pairwise dot plots, 20 different dot plots are generated, excluding FSC and SSC plots. Since spermatozoa are incubated under three different conditions in the experiment, we have 60 different dot plots. To improve data visualization, techniques such as t-SNE maps can be used. T-Distributed stochastic neighbor embedding (t-SNE) is an unsupervised self-learning algorithm for dimensional reduction, which primary use is the visualization of large data sets, as originated by flow cytometry [8]. The t-SNE algorithm utilizes a gradual iterative approach to find a lower dimensional representation of the original data while preserving the original information of the data set. In terms of flow cytometry data means that we can visualize in a single plot all the possible combinations of fluorochromes in a multiparametric analysis. Simultaneous analysis and visualization of all the parameters in a multicolor flow cytometry experiment offer interesting new information on the nature of the mammalian ejaculate, as several recent reports demonstrate the value of this approach in spermatology [9, 10]. Particularly interesting is the identification that capacitation only occurs in a subpopulation of acrosome intact spermatozoa and that this subpopulation shows decreased intracellular Na^+ and increased membrane polarization (Em) [9, 10]. Our laboratory investigated changes induced by cryopreservation in stallion spermatozoa [5, 11]. Conventional and computational flow cytometry using t-distributed stochastic neighbor embedding (t-SNE) show that most changes induced by cryopreservation are apoptotic, including an increase in caspase 3 activation, PS translocation to the outer membrane, loss of mitochondrial membrane potential, and increase in intracellular Na^+. Average values of markers of capacitation-like changes were not affected by cryopreservation; however, the analysis of the phenotype of individual spermatozoa using computational flow cytometry revealed the presence of subpopulations of spermatozoa experiencing capacitation-like changes.

In sum, the increasing complexity of flow cytometry analysis is providing interesting outcomes on the biology of the stallion spermatozoa. More advanced flow cytometers facilitate the use of multicolor panels generating huge amounts of data that may require the use of advanced techniques. All these aspects put together are expanding enormously the possibilities of flow cytometry in the study of sperm biology, leading to a growing subfield of flow cytometry that can be termed flow spermetry.

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CONFLICT OF INTEREST STATEMENT

The author does not have conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

This is a commentary article, and relevant data are included in the manuscript.

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