Review Report (invited)

Clinical applications of flow cytometric sperm analyses

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Abstract

Animal andrology has benefitted by flow cytometry use (to analyze various sperm components). Examples include its use to explore DNA intactness, to sort sperm for chromosomal sex, or to explore via suitable fluorophores the functional intactness of essential attributes (e.g. plasma membrane). These explorations can provide the clinician with evidence of the extent of cell death, a major variable in using semen for breeding. Such gains have evolved over the past decade(s) through the production of versatile markers, including biomarkers, the design of simpler protocols, and the availability of more user-friendly and less expensive bench-flow cytometers. The present review summarizes the current state of flow cytometry use within animal andrology. It attempts to be critical of research gains, separating them for clinical relevance and focusing on its use to examine the early events that occur in the labile sperm plasma membrane (leading to cell death) – a most relevant marker for clinical decisions during diagnosis and prognosis of fertility when using ejaculated sperm (either unprocessed or processed) for cooling, freezing, or sperm sorting for in vitro fertilization or artificial insemination.

Keywords: Semen analysis, stallion, flow cytometry, sperm membrane, livestock

Introduction

Clinical veterinary andrology attempts to assess the health of a breeding male. It certainly includes examination of genital organs, the ability to mate, and a thorough examination of collected semen, pursuing a species-specific general breeding soundness evaluation.[1] Decades of animal selection and dedicated clinical work have yielded substantial knowledge for early identification of potentially infertile sires, preventing their use for the widespread and most successful reproductive biotechnology, artificial insemination (AI). Furthermore, laboratory examination of semen has provided steady support for accurate diagnoses, particularly when novel methods and equipment have been presented.[2] Despite methods for assessing semen normality and, particularly, sperm structure and function have been a target for andrological and spermatological research, the conventional evaluation of the ejaculate made by most clinicians has been restricted to determinations of sperm number, motility, and very seldom, morphology.[3–5] This slim battery of semen parameters has historically been considered sufficient to potentially separate fertile from infertile.[6] Yet, the proportion of subfertile sires is still sufficiently high to cause losses on the level of billions of Euros or Dollars, yearly. These losses are often recognized too late, either when conception is clinically assessed, when nonreturn figures are indirectly measured, or when offspring is absent or lower than expected. Although sire fertility is evidently a limiting factor, most fertility research seldom focuses on the male or the quality of the used sperm.[6] One of the reasons behind is that although sperm biomarkers related to fertility exist, their use by the clinician is still burdened by requirements of high technical know-how, expensive instrumentation, and the inability of the researchers to communicate their diagnostic value, mostly due to insufficient standardization of valuable protocols since the use flow cytometry is becoming common in established companies.[6] Another factor to consider is the complex nature of fertility depending on numerous factors and their interactions.[2,6,7]

Innumerable methods are now available for semen/sperm evaluation, including those examining the capability of sperm

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to interact with the surrounding fluids (seminal plasma, intralumenal female genital fluids and extension artificial media we use for preservation, in vitro culture, and sorting), the lining epithelia of the internal female tract, or the coatings of the oocyte.[8–11] Currently, we can explore not only sperm plasma membrane status, various sperm organelles, sperm genome, and its transcriptome but also the capacity of sperm to fertilize and to initiate early embryo development in vitro.[12–17] In addition, we are helped by several computer-assisted motility analyzers, of morphology screeners, and by flow cytometers of various complexity (and cost), including cutting-edge image-based fluorescence correlation spectroscopy for high-throughput cell image acquisition.[6,18–20] Authors advise the readers to be acquainted with some of the excellent reviews available to grasp this development.[20,21] For the clinician, perhaps the most relevant take-home message is to remember that sperm are highly differentiated cells, programmed to interact and react to the environment and prompt to suffer changes that inexorably lead to sperm death, and hence inability to participate in fertilization, and perhaps, to cause deleterious changes in the accompanying sperm.[22] Our intention with this review is to remind the clinician of the basic and essential diagnostic values of determining the percentages of live sperm in the sample for its fate. This concept that calls for methods that can identify sperm changes before death is established, considering sperm death is irreversible. Although the integrity of the plasma membrane is rapidly lost when necrotic cell death occurs, subtle changes characterize other forms of sperm demise. Numerous flow cytometry (FC) protocols have been developed to identify necrotic sperm and also various degrees of plasma membrane alteration occurring prior to sperm death.[23–27] In this paper, we provide an update on the various forms of death in sperm, and how these forms are reflected on various FC protocols, establishing a critical review of the current methods for the determination of the integrity and functionality of sperm plasma membrane. A particular call is made for the value of using multiparametric FC that, aided by computational analysis (Cytobank), can simultaneously identify multiple forms of sperm damage. Although these multiple commercial bench-top FCs are available, the authors recommend the use of modern equipment with upgrade possibility (e.g. activation of new lasers), and having a friendly and open software.

Necrosis/apoptosis/ferroptosis/spermptosis/ premature capacitation

Although the existence of classical necrosis in sperm is widely accepted, the presence of various forms of programmed cell death has been under debate. The transcriptionally silent nature of sperm has been claimed to make programmed forms of cell death unlikely in these cells; however, currently, it is considered that sperm are programmed for cell death as a measure to promote the silent removal of redundant sperm for the female reproductive tract.[28] Moreover, although protein synthesis in sperm is negligible, the incorporation of new proteins through microvesicle trafficking and, especially, posttranslational modifications of existing proteins modulate sperm function. Despite all sperm are activated through cell death, this may occur at various velocities and factors, i.e. oxidative stress, triggering and accelerating the process. Although the terms programmed cell death and apoptosis have been used indistinctly, these terms are not synonymous. Various forms of programmed cell death may share similar molecular mechanisms; however, all these forms are not identical. Necrosis is characterized by a rapid loss of membrane integrity. However, in various forms of programmed cell death, more subtle changes occur, especially in a cell as highly specialized as sperm. Capacitation is the maturational process that sensitizes sperm to recognize and fertilize the oocyte; this process causes substantial plasma membrane modifications. Capacitation involves the removal of cholesterol from the plasma membrane, removal of coating materials from the membrane, a rise in intracellular Ca^{2+}, an increase in intracellular cAMP, and a dramatic increase in tyrosine phosphorylation. Removal of cholesterol from the membrane is preceded by its oxidation, stimulated by bicarbonate, and the formation of oxysterols,[29–31] which are depleted from the sperm membrane by albumin. The process prepares the sperm to fertilize an oocyte; however, most of the sperm in the ejaculate will never reach an egg and will die within the female reproductive tract and only a small subpopulation of sperm fully capacitate,[32,33] thus mechanisms for immunologically silent elimination of redundant sperm are likely. Capacitation shares molecular mechanisms with what begins to be considered special forms of programmed cell death in sperm: redox-regulated, intracellular Ca^{2+} increase and membrane destabilization occur. However, capacitation implies a dramatic increase in tyrosine phosphorylation and changes in the pattern of motility to hyperactivated motility, whereas programmed cell death ends with cessation of any kind of motility in sperm. Finally, apoptotic sperm release 'find-me/eat me' signals to achieve a silent removal of redundant sperm. The inhibition of glutathione peroxidase 4 (GPX4) leads to a specific form of cellular demise termed ferroptosis.[34] This is a form of programmed cell death that is characterized by the failure of glutathione (GSH)-dependent antioxidant defenses that can be triggered by the inhibition of GPX4 or SLCTA11/xCT, the antiporter that exchanges extracellular cystine for intracellular glutamate. Cystine is used for GSH synthesis, a mechanism recently described in stallion sperm.[27,35] It is possible that this form of cell death is present in sperm.[26]

Induction of apoptosis or necrosis in sperm

If the presence of apoptosis in ejaculated sperm is a remnant of spermatogenesis (as stated by the abortive apoptosis theory), most sperm are already programmed to experience programmed cell death; thus, the concept is that more than induced, in sperm, the programmed cell death process can be accelerated. However, classical inductors of apoptosis have been successfully used in stallion sperm, although betulinic acid seems more effective to induce apoptosis than staurosporine or thapsigargin,[36] which is in agreement with the predominance on the mitochondrial pathway of apoptosis in sperm.[37] In sperm, cryopreservation has been constantly associated with the production of apoptotic changes related to the oxidative stress caused by freezing and thawing.

Changes in the integrity of the plasma membrane

Although the integrity of the plasma membrane is rapidly lost when necrotic cell death occurs, subtle changes occur under other forms of sperm demise. The traditional approach for the determination of viability in sperm biology has been using dye exclusion assays; the combination of the permeable DNA binding probe SYBR-14 and the impermeable DNA binding probe propidium iodide is widely used and is marketed as a kit for sperm viability assay. This assay became very popular, especially in the veterinary world, since its introduction in the early 90s of the past century. This combination was appropriate for the single laser (blue) of the cytometers commonly

used in the andrology labs at that time. During the first decade of the present century, more sophisticated assays started to be introduced in spermatology; these techniques allowed to disclose distinct physiological states of the membranes in addition to necrotic cells, and these assays identified the permeability of the membranes, the order of the lipid packaging, the transposition of specific phospholipids, the polarity of the membrane, and assays able to identify the peroxidation of membrane lipids.

Assays identifying changes in membrane permeability

The probe that has gained popularity in the spermatology field is YoPro-1. Initially, this probe was used as an alternative to red emission viability dyes, when merocyanine-540 was used.[38–40] However, YoPro-1 not only is able to stain necrotic cells but can also use specific channels to cross intact membranes.[41–43] Moreover, live sperm may pump out YoPro-1, whereas depletion of ATP may result in accumulation of YoPro-1 in the sperm.[44] This property allows the identification of sperm, with intact membranes, but in a compromised physiological situation; therefore, this dye is used to identify apoptotic cells and to identify increased membrane permeability due to lipid peroxidation. An interesting report uses the YoPro-1 assay to sort sperm of better quality (low DNA fragmentation) in human sperm since DNA fragmentation of the DNA in sperm is due to oxidative attack, sperm experiencing oxidative stress also have increased membrane permeability,[45] and these sperm can be easily identified and removed.[46]

Lipid peroxidation of the membrane increases membrane permeability: Assays identifying the transposition of phospholipids

Among the changes that oxidative stress induces is the increased permeability of the plasmalemma.[45] Lipid peroxidation is thus the consequence of oxidative stress and is a chain reaction, with peroxidation products created close to each other. The identification of the transposition of specific phospholipids from the inner to the outer membrane constitutes a classical assay for the detection of apoptotic cells. The Annexin-V assay is a popular technique to identify the presence of phosphatidylserine (PS) in the outer leaflet of the plasmalemma. Under normal conditions, PS and phosphatidylethanolamine are present in the interleaflet of the plasmalemma. When the cell enters apoptosis, PS translocated to the outer membrane constituting an 'eat me' signaling (presence of PS signals the cells that will be phagocytosed in a silent, noninflammatory manner). This is especially important for the elimination of redundant sperm from the female genitalia, and failure in this mechanism may lead to an uncontrolled inflammatory reaction to semen and posterior endometritis.

Polarity of the plasma membrane

An anionic bis oxonol dye (DiSBAC2)[47] enters depolarized cells where they bind to the intracellular proteins of membranes and exhibit enhanced fluorescence and red-orange spectral shifts. Increased depolarization results in more influx of the anionic dye, and thus an increase in fluorescence. Conversely, hyperpolarization is indicated by a decrease in fluorescence. In contrast to cationic carbocyanines, anionic bis oxonols are largely excluded from mitochondria and are primarily sensitive to plasma membrane potential.[48] The use of flow cytometry allows determining, at the single cell level, many distinct characteristics of sperm. Recently, changes in the potential of the membrane have been studied in relation to capacitation and cryopreservation.[33,49] These changes relate to modifications in intracellular Na+ that can also be monitored using flow cytometry. The potential of the sperm membrane can be determined with the probe DisBAC2 combined with a vitality dye to exclude dead sperm from the analysis. Depolarization of the membrane has been reported in relation to apoptotic changes,[49] whereas capacitation is linked to hyperpolarization.[33] Interestingly, this occurs only in a subpopulation of live, intact acrosome sperm and not in the whole ejaculate. Depolarization of the membrane has been related to apoptotic changes in somatic cells and in sperm. Thus, combined staining has been employed to determine if depolarized sperm correspond also to sperm having apoptotic features. These experiments demonstrate that depolarized sperm are also caspase 3 positive, express PS translocation, and have increased intracellular Na+ and collapsed mitochondria.[49]

Active sperm caspases: denouncing sperm senescence

Senescent sperm express active caspase 3.[37,50,51] Depending of the presence of prosurvival factors, caspase 3 remains inactive due to the phosphorylation of protein kinase B (PKB or Akt).[43,51,52] If prosurvival factors are lost or oxidative stress reaches a threshold, caspase 3 is activated and sperm senescence and death are triggered.[37,53] Cryopreservation triggers this phenomenon, and surviving sperm experience accelerated senescence.[52,54–57] Active caspase 3 can be detected using CellEvent Caspase-3 Green Detection Reagent, which consists of a four-amino-acid peptide (DEVD) conjugated to a nucleic acid-binding dye. This cell-permeant substrate is intrinsically nonfluorescent because the DEVD peptide inhibits the ability of the dye to bind to DNA. After the activation of caspase 3 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response with an absorption/emission maxima of 502/530 nm.

Flow cytometry data output and clinical interpretation

An example (Figure 1) of a multiparametric panel as an output of stallion sperm studied with a modern flow cytometer (Cytoflex LX), providing data relevant for the clinician to evaluate the current and potential status of a semen sample. Description of this four-color experiment implies that the information provided has to be depicted in several dot plots since the number of 2D plots increases exponentially with the number of colors in the analysis. Additionally, the establishment of gates is performed manually. These are confounding data for a nonspecialist and ought to be simplified without losing relevant information, for instance, using self-learning artificial intelligence techniques. An example of such is presented (Figure 2), using the Flo self-organizing maps for visualization and organization of flow cytometry data. With this technique, all the characteristics of the sperm in the sample can be visualized at a glance, facilitating comparisons among samples and identification of subtle changes. In the example, aliquots of the same stallion ejaculate were extended and incubated at 37°C for 3 hours in 2 distinct media. Sperm were stained with a 4 colors protocol, to identify live, apoptotic (Caspase 3 and Annexin V) and necrotic sperm (Live/Dead Viakrom 808®). A quick view of this panel, comparing the 2 incubation sets (1 versus 2), allows the observer to see that there are more viable sperma in 1a than in mapping 2. In the

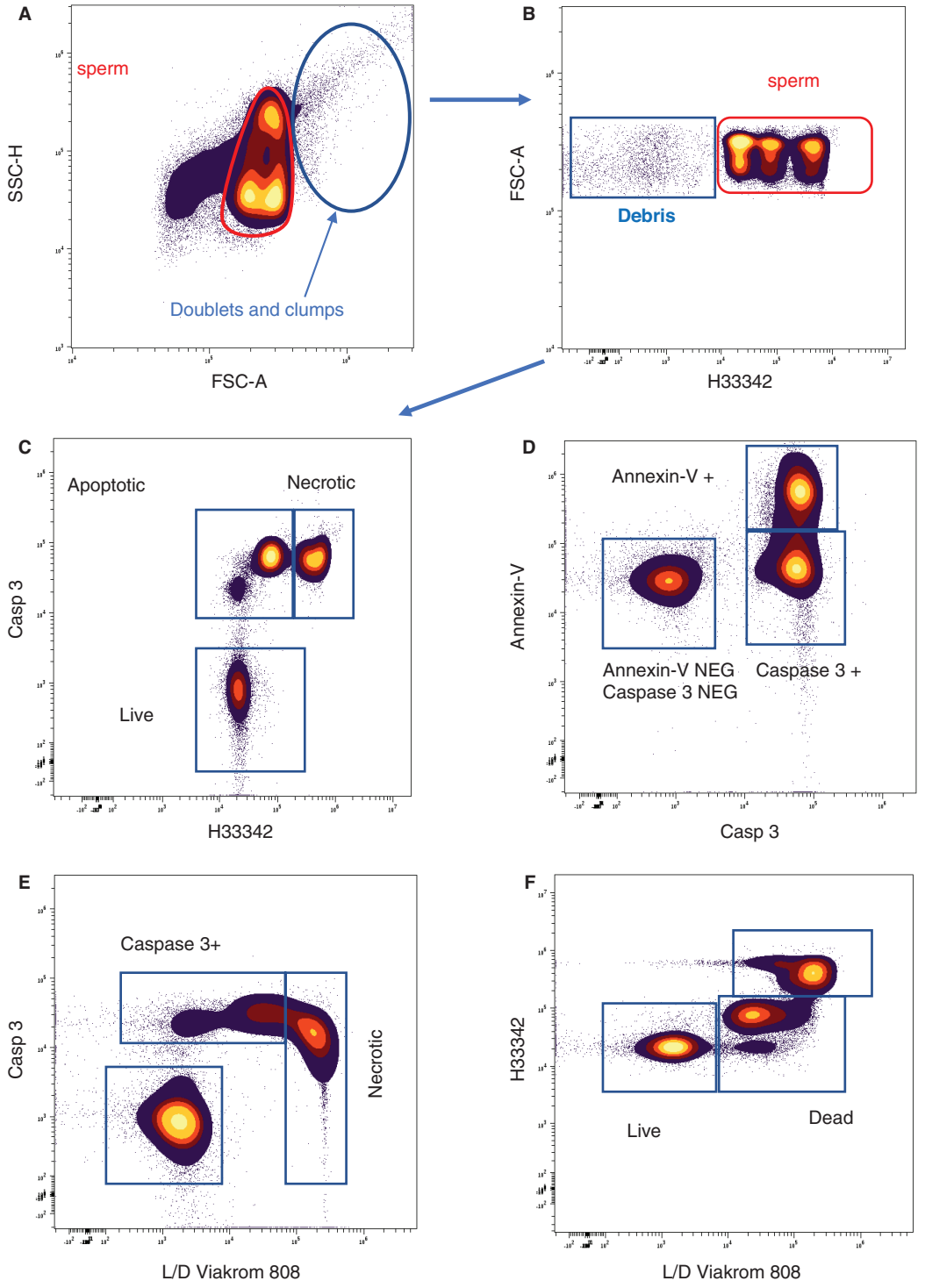

Figure 1. Example of a multiparametric panel for the study of stallion sperm. (A) Gate for the sperm, where sperm events are identified based on forward (FSC) and side scatter; events having higher area in the FSC-A are removed from the analysis since they represent doublets and clumps. (B) To remove debris, the sample is stained with a permeable DNA binding probe (Hoechst 33342), and only DNA bearing particles, mostly sperm, are stained. Unstained particles (debris) are removed from the analysis. (C) Combination of H33342 and CellEvent®; this combination of probes identifies 3 sperm populations corresponding to live, apoptotic, and necrotic sperm. (D) Double staining with 3 probes to identify apoptotic changes, CellEvent®, to identify Caspase 3 positive sperm and Annexin-V to identify sperm having transposition of phosphatidylserine to the outer leaflet of the membrane. Three populations are easily identified. (E) Combination of CellEvent®, and the fixable live/dead probe Viakrom 808®. This combination easily identifies 3 populations: live, apoptotic, and necrotic sperm. (F) Combination of H33342 and the fixable live/dead probe Viakrom 808®; this combination of probes identifies 3 types of populations: live sperm, dead sperm, and damaged sperm (2 & 3 positive for Viakrom 808®).

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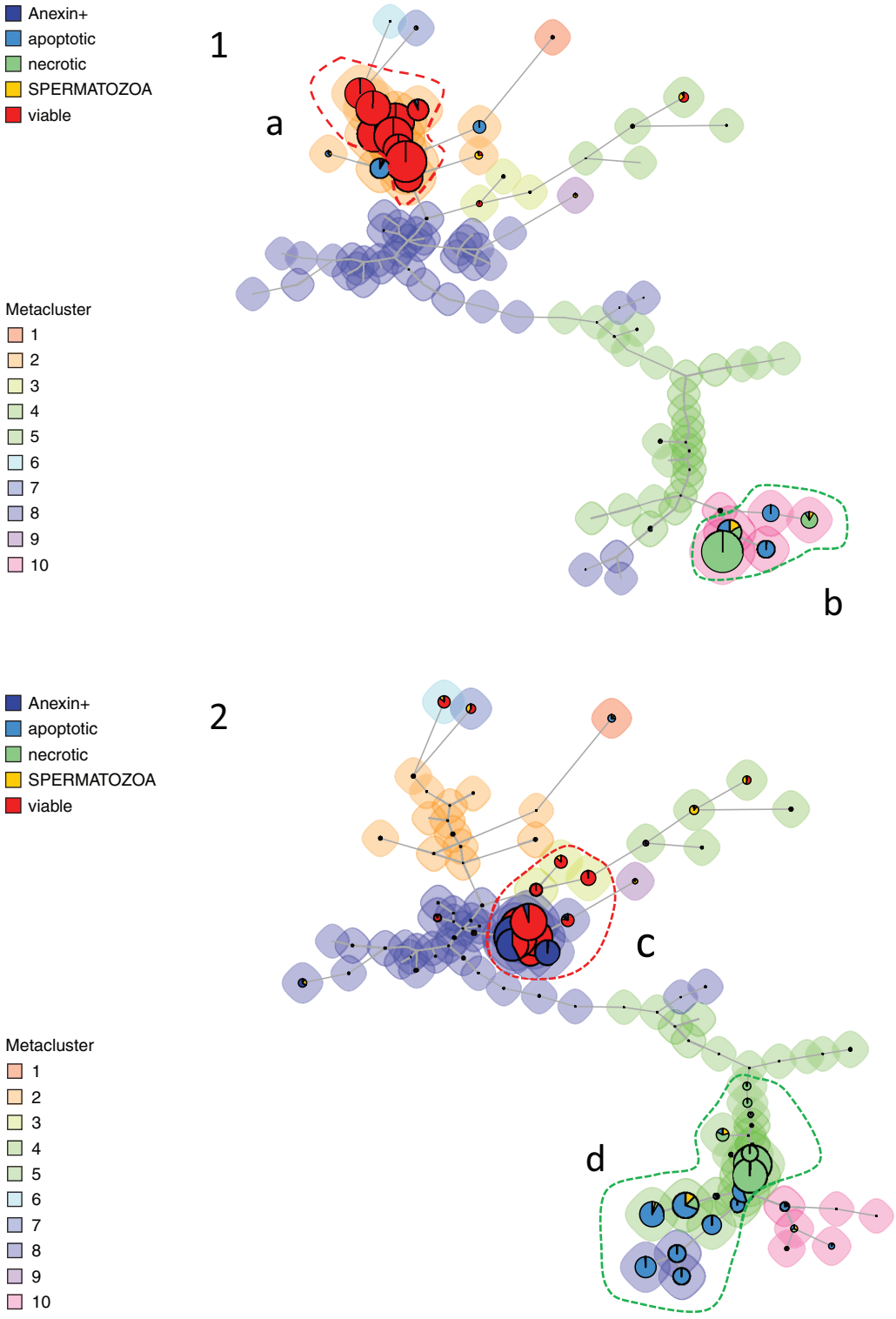

Figure 2. Use of artificial intelligence techniques to simplify the output of flow cytometry analysis. Figure presents a Flo self-organizing mapping for visualization and organization of flow cytometry data where all the characteristics of stallion sperm in aliquots of the same ejaculate, extended, and incubated at 37°C for 3 hours in 2 media (1 versus 2) are presented and visualized at a glance. Here, sperm were stained with a 4 colors protocol to identify live, apoptotic (Caspase 3 and Annexin V), and necrotic sperm (Live/Dead Viakrom 808®) in particular clusters. The gains in time are relevant, for instance, after live sperm (red circles) comparing the 2 incubation panels (1 versus 2). In 1a, there are more viable sperm than in panel 2, where there is a larger abundance of sperm having several apoptotic markers. Interestingly as well, panel 2 depicts the presence of many live sperm having phosphatidylserine transposition to the outer membrane, representing unstable membranes.

latter, sperm having various apoptotic markers are more abundant (see 2d). Moreover, many live sperm exhibit phosphatidylserine transposition to the outer membrane, representing unstable membranes. Such sperm are at risk, and the clinician benefits from knowing all this battery of data and its quantification before deciding the fate of the ejaculate or the use of a processed sample for further use. Hopefully, there shall be gains in diagnostics and in resulting fertility.

Concluding remark

Cytomics, combining novel designed motility analyzers, multiparametric flow cytometers, and enhanced digital imaging together with the incorporation of artificial intelligence in the analysis, shall dominate the landscape of andrological laboratories and enable quick determinations on huge sperm numbers for markers highly relevant to sperm function and, hence, for fertility. Such advancements shall, however, hardly replace essential clinical andrological practice.

Ethical permission

Housing, handling, and collection of semen that have been used during the analyses described in this review have followed the European Community Directive 2010/63/EU, 22/09/2010, and current Spanish (Law 6/2913 June 11th and European Directive 2010/63/EU) and Swedish legislation (SJVFS 2017:40). Studies involved had previously been scrutinized and approved by the Ethical Committee of the University of Extremadura (PID2021-122351OB-I00 and IB20008) and the Swedish Regional Committee for Ethical Approval of Animal Experiments (Linköpings Djurförsöksetiska nämnd, Linköping, Sweden, Permit numbers 75-12 (10/02/2012), ID1400 (02/02/2018), and Dnr 03416-2020 (26/03/2020)).

Conflict of interest

Authors declare no (financial, commercial, or personal) conflict of interest.

Author contribution

Authors contributed equally to the design and writing.

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